DECOMPOSITION OF THE LACTOSE OPERON

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Abstract

A system was established which would allow the analysis of the Lactose Operon of *Escherichia coli* (*E.coli*) using Metabolic Control Analysis (MCA). The two main constituent parts of the operon i.e. the *lacZ* and *lacY* genes were separated to permit the independent modulation of their expression. The *lacZ* gene retained the *lac* controlling sequences and the *lacY* gene was transferred to the controlling sequences of a different inducible promoter. The promoter chosen was the *trp* operon promoter, which is inducible with the addition of antirepressor.

The *trp* promoter/ *lacY* construct included regions of the *E.coli trp* operon biosynthetic genes, *B* and *A*, flanking the *lacY* construct. The flanking regions were employed to integrate the construct into the chromosome of an *E.coli* strain with a deletion in *lacY*. Two methods were used to achieve this homologous recombination

(1) Transformation of a suitable *recD* recipient strain with linear DNA and

(2) Specialised Transduction with Bacteriophage lambda (λ).

The second method yielded strains carrying the *lacY* construct in a stable integrated copy within the *trpB* gene, generating a trp^- mutation through insertion.

The constructed strain grew on lactose and displayed permease activity.

The expression of the *lacY* gene was assayed and was demonstrated to be induced with the addition of the antirepressor IAA and to be independent of *lacZ* expression.

It is possible therefore to modulate β-galactosidase activity in this strain using IPTG and to modulate permease activity using IAA.

Declaration

This thesis was composed by myself and describes my own work except where otherwise stated in the Acknowledgements or in the text.

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Abbreviations

A_{260}	Absorbance at 260nm
ACH	Casamino acids
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pairs
$CHCl_3$	Chloroform
CIP	Calf Intestinal Phosphatase
cpm	counts per minute
CsCl	Caesium Chloride
dATP	deoxyadenosine triphosphate
(α-^{35}S)dATP	(α-^{35}S) deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
(α-^{32}P)dCTP	(α-^{32}P)-2-deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanidine triphosphate
ddTTP	dideoxythymidine triphosphate
DEAE	Diethyl amino ethyl
DEPC	Diethyl pyrocarbonate
dGTP	deoxyguanidine triphosphate

dH_2O	distilled water
DIG	Dioxygenin
DMF	N, N, Dimethyl formamide
DMSO	Dimethyl sulphoxide
dNTP	deoxyribonucleotide triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dTTP	deoxythymidine triphosphate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethelenediamine-tetra-acetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
g	gravity constant
IAA	3-β-indole-acrylic acid
IPTG	isopropyl-β-D-thio-galactopyranoside
kb	kilo bases
lac	lactose
log	logarithm
LMP	low melting point
μg	microgram

μl	microlitre
mA	milliamperes
MES	2-[N-morpholino]-ethanesulfonic acid
mg	milligram
ml	millilitre
ng	nanogram
ONPG	o- nitrophenyl-β-D-galactopyranoside
PEG	Polyethylene glycol
RNAse	ribonuclease
S. marcescens	*Serratia marcescens*
SDS	sodium dodecyl sulphate
TEMED	N,N,N'N'-Tetramethylethylenediamine
TONPG	o-nitrophenyl-β-D-thio-galactopyranoside
Tris	Tris(hyroxymethyl) methylamine
trp	tryptophan
UV	ultraviolet
V	volts
v/v	volume by volume
w/v	weight by volume
MES	

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1 Introduction

1.1 General Introduction

Traditionally, geneticists and biochemists have approached the study of metabolic regulation from very different viewpoints. Typically, the biochemists' approach has been to study metabolic pathways by examining the properties of individual enzymes in isolation from the system i.e. purifying the enzyme measuring its K_m and Vmax *in vitro* under different conditions and so on. From such studies several methods of regulation of enzyme activity have been proposed e.g. cooperativity, feedback inhibition and activation by phosphorylation. Geneticists conventially view an organism's *in vivo* metabolism as a black box through which genetic information is translated into the phenotype. Consequently only differences in phenotype of the organism and the mutations which cause them have been of interest.

More recently some biochemists/geneticists have attempted to combine both approaches by analysing intact systems. A number of groups have developed alternative theoretical approaches to allow a quantitative analysis of metabolic regulation of intact systems rather than a qualitative study ; Higgins (1965) [44]; Savageau (1972) [101]; Kacser & Burns (1973) [59]; Heinrich & Rapoport (1974) [40]; Crabtree & Newsholme (1985) [21, 20].

Of these approaches some are more practicably applicable to experimental systems than others. Metabolic Control Analysis (MCA) [111, 59, 13], as proposed by Kacser & Burns and Heinrich & Rapoport, has been the most

successfully applied and, as a result, the most widely used.

MCA provides a means of analysing in a quantifiable way the complex relationships involved in a cell's metabolism and provides the means to relate observations made at the genotypic level, *in vitro*, to observations and new experimental explorations made at a phenotypic level, *in vivo*. Thus, it can offer explanations of phenomena such as dominance, recessivity, epistasis and pleiotropy [62, 57, 58, 59] as well as real insight into the regulation of metabolic pathways. In short, it allows the system as a whole to be examined.

1.2 Metabolic Control Analysis

An organism can be thought of as an enzyme system consisting of many different diverging and converging pathways: each step of which is catalysed by a specific enzyme. The enzymes in such pathways do not work in isolation but are kinetically coupled to one another through their substrates and products. The relationship of one enzyme to another results in the net flux across any enzyme step being affected by the activities of its neighbouring enzymes. The flux through each part of the metabolic map is therefore dependent on the kinetic parameters of all the enzymes and hence on the genetic specification of the whole genome. Measuring the effect of varying the activity of an enzyme *in vitro* will normally generate a linear response curve. (see Figure 1, curve 1) As a result of kinetic coupling however, the effects of changing the catalytic activity or enzyme concentration at one step, tend to be buffered by the re-

sponse to this at other steps. In genetic terminology, genetic systems have built in epistasis. The effect of variation in the same enzyme when measured *in vivo* will generate a non-linear response (see Figure 1, curve 2). This is due to the tendency of any effect, in general, to be buffered as it spreads through the system.

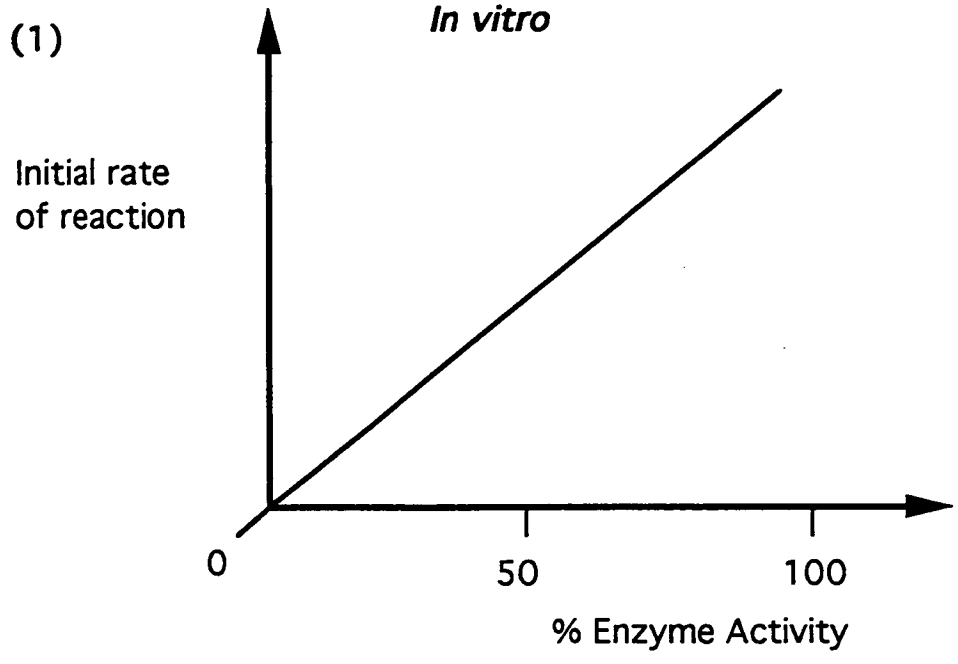
Proponents of MCA, therefore, hold that the effect on the flux of altering the genetic specification of a single enzyme cannot be accurately measured *in vitro* but only *in vivo* where the response of the system as a whole can be observed.

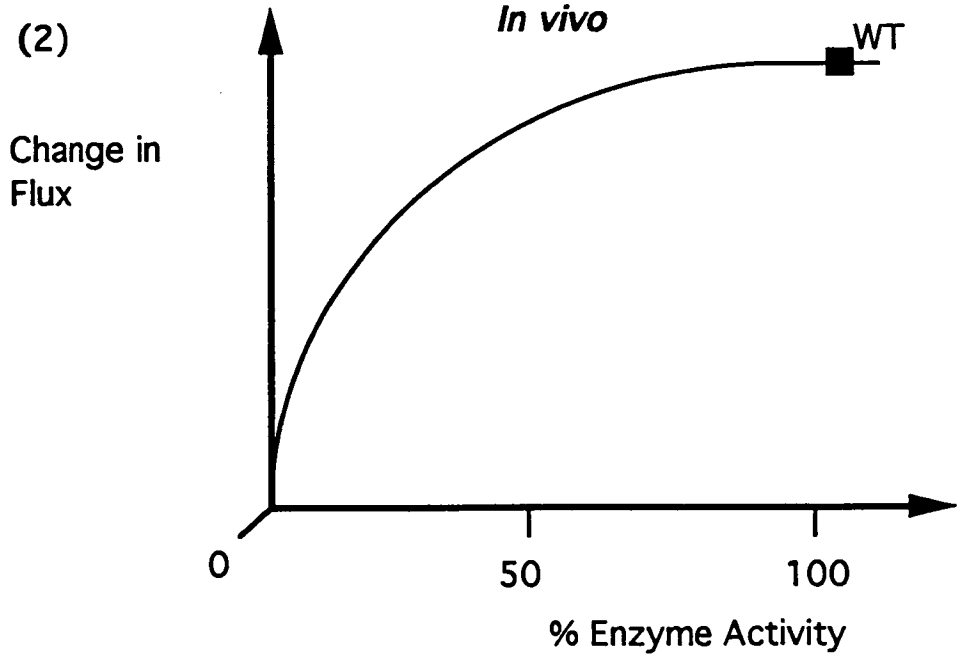
Figure 1: Typical Enzyme curves when assayed *in vitro* and *in vivo*

(1)A schematic representation of a typical linear response curve measuring the effect of variation in enzyme activity on the initial rate of reaction *in vitro*.

(2)A schematic representation of a typical hyperbolic response curve measuring the effect of variation in enzyme activity on the flux through the pathway *in vivo*.

Figure1:: Typical Enzyme curves when assayed *in vitro* and *in vivo*





1.2.1 The Control Coefficient

The enzyme system to be studied is defined by the experimenter [45] and can be described in the form of a series of constant and variable quantities. Constant quantities, or parameters, are those which do not change unless affected from outside the system e.g. external pools, external ligands and the kinetic constants of each enzyme catalysed step. The parameters of the system, along with initial conditions and time, define the state of the system. System variables are those which are dependent on the state of the system e.g. metabolite concentrations and pathway fluxes.

The measurement of the response of the system (Effect) to a specific change (Cause) of a system parameter requires that all other parameters are held constant. To achieve this "cause" and "effect" scenario it is easier to make comparisons under steady state conditions i.e. identical external conditions and when all the intermediate metabolite pools and all the fluxes in other parts of metabolism are constant with time. The induced change will reverberate through the whole system until a new steady state has been reached. The change in the variables of the system i.e. fluxes/metabolite concentration can then be measured as a direct consequence of the change in the parameter of interest.

For example, the ratio of the change in the variables of the system versus the change in enzyme activity gives a measure of the ability of the enzyme to

alter the system e.g.

Influence on Flux $= \frac{\Delta J_j}{\Delta E_i}$

if "fractional" changes are used to eliminate arbitrary units, equation (1) can be modified to give:

Influence on Flux $= \frac{\Delta J_j}{\Delta E_i} \times \frac{E_i}{J_j}$

As a consequence of the non-linearity of the response curve, (Figure 1), the influence on flux must be measured as the tangent of flux versus enzyme activity at the point of interest. Therefore, if the change in enzyme activity is very small and hence $\Delta E_i \rightarrow 0$, the " *Influence on Flux* ", which in this case defines the Control Coefficient [13, 59] can be written as;

Influence on Flux $\equiv C_i^J = \frac{\partial J_j}{\partial E_i} \times \frac{E_i}{J_j}$ = Slope x Normalising Factor

where C_i^J is the control coefficient measuring the change in flux (J_j) with respect to the induced change in enzyme (E_i).

Such infinitessimal changes are impossible to impose, and to measure experimentally. Traditionally, therefore, to obtain an experimental estimate of a Control Coefficient, small finite changes have been used to draw, and by extrapolation and/or intrapolation, a response curve around a particular point obtained.

1.2.2 The Summation Theorem

Rigorous algebraic derivations and experimental observations from these basic principles have confirmed the arguments discussed previously. In addition one of the most important relationships to come out of MCA is the finding that the sum of all the flux control coefficients for all the different steps in a pathway flux is equal to unity [59, 40] i.e. For a system with n enzymes;

$$\sum_{i=1}^{n} C^{J}_{E_i} = 1 \tag{1}$$

This holds for any flux chosen. In any real system, the summation theorem predicts that the values of individual control coefficients are likely to be very small. The general expectation is that no one enzyme in a pathway is likely to control the flux but that all the enzymes share the control. A more realistic view, perhaps, is that there will be a distribution of control such that a few enzymes in the pathway hold the majority of control while the other enzymes have little or no effect. Another intrinsic property of the summation theorem is that changing the flux control coefficient at one step must result in a compensating change in the values of all the other control coefficients although their enzymes have not been changed.

The magnitude of the flux control coefficient represents the "importance" of a particular enzyme in its potential to influence flux at the operating point of the system i.e. a particular steady state. It therefore gives a precise measure

of a particular enzyme's contribution when acting within the system. This knowledge enables the experimenter to understand the enzyme relationships *in vivo* and, if desired, to choose which enzyme(s) to manipulate in order to have the maximum effect on flux. Criteria used in previous studies to determine the choice of enzyme, such as those which catalyse non-equilibrium reactions or "rate limiting" steps have rarely been successful and have no basis in theory. For example, studies using multi-copy vectors carrying genes for various enzymes carried out on the glycolytic pathway in *Saccharomyces cerevisiae* have demonstrated that the enzymes, previously believed to be "rate limiting", phosphofructokinase (PFK) and pyruvate kinase (PK), have very little influence on flux through the pathway under anaerobic conditions [50].

Several examples exists which demonstrate the usefulness of MCA. Starch metabolism in round (RR or Rr) and wrinkled (rr) peas has been examined. In particular, the effect of variation in the starch branching enzyme on a number of other metabolic parameters has led to an understanding of how these phenotypes occur [10]. These experiments demonstrated that Mendel's original morphological mutants resulted from enzyme changes. The control of aromatic amino acid catabolism, in rat hepatocites, has also been addressed and control cofficients have been measured successfully for several enzymes in this pathway [100]. A major conclusion of the experiments was that the enzymes described as "rate-limiting" for their respective pathways were not solely responsible for

controlling the flux but that the control was distributed over the pathways. The transport of the three aromatic amino acids across the plasma membrane which had previously been overlooked, was demonstrated to be very important in the control of amino acid catabolism and the synthesis of biogenic amines in the central nervous system.

This thesis describes the establishment of an experimental system to allow Metabolic Control Analysis to be applied to the the classic model of metabolic regulation, the *lactose* operon in *Escherichia coli.*

1.3 The Lactose Operon in *E.coli*

Most of the major concepts of operon structure, expression and regulation have come out of work on the lactose (*lac*) operon in *E.coli* [52, 63, 51]. Convenient aspects of the chemistry, physiology and genetics of lactose metabolism in *E.coli* make it a particularly profitable system to analyse. Consequently the *lac* operon has been studied thoroughly, its genetics and molecular biology are well characterised. However, much work still needs to be carried out to understand the physiology of lactose metabolism i.e. the question of the consequences to the cell of how the operon acts *in vivo* has still to be answered.

Jacob and Monod used the system of lactose metabolism in *E.coli* to examine the problem of enzyme adaption, characterised as the appearance of a specific enzyme only in the presence of specific substrates. Genetic and bio-

chemical studies yielded a simple model for the regulation of the *lac* operon and this is used as a classical example of gene expression and regulation [53].

1.4 The Genes and Gene Products of the Lactose Operon

Although most of the details described below are well known, they are set down because they influenced the experimental strategy of the cloning experiments.

An operon can be defined as an arrangement of related structural genes, in groups of two or more, which share a single promoter and terminator. Additional controlling elements, such as operators, are usually associated with operons. The *lac* operon itself is made up of three structural genes, all sharing a single promoter/operator sequence.The entire operon has been cloned and sequenced [37, 61, 12, 39].

The cluster of genes are transcribed into a single mRNA from which the individual proteins are translated [52, 51, 63, 53]. The structural genes *lacZ, Y* and *A* code for a β-galactosidase, a permease and a thiogalactoside transacetylase respectively. The role each of these proteins play in lactose metabolism has been characterised. The permease catalyses the transport of lactose into the cell, lactose diffuses across the outer cell membrane but is actively transported across the cytoplasmic membrane. β-galactosidase catalyses the breakdown of the internalised lactose molecule into its constituent parts i.e. glucose and galactose to be metabolised further within the cell. The role of the thiogalactoside transacetylase is the least clear but some evidence exists which suggests

the enzyme is involved in detoxification. The enzyme has been shown to confer a selective advantage on cells grown in the presence of nonmetabolisable analogs of lactose [2]. Transacetylase activity does not appear to be part of the metabolism of lactose to growth.

Control of the operon is mediated via the *lac* repressor protein, encoded by the *lacI* gene which is situated close to the *lac* operon, on the *E.coli* chromosome. In the absence of lactose the repressor protein binds to the promoter/operator region of the *lac* operon and prevents transcription of the *lac* genes. The *lacI* gene has also been cloned and sequenced [32] and has its own promoter and terminator sequences forming an independent transcription unit.

1.4.1 The *lac* Promoter/Operator

The promoter sequence is required for the initiation of transcription of the *lac* operon. The promoter sequence contains three promoter elements P1, P2 and P3 as well as information necessary for the specific interaction of two proteins with the DNA, RNA polymerase and the Catabolite Activator Protein (CAP). The major promoter is P1. P2 and P3 are two upstream promoter like elements which only direct the synthesis of very low levels of *lac* mRNA *in vivo* [74, 75, 49].

Transcription initiation is thought to occur when CAP, in the presence of adenosine 3′, 5′-monophosphate (cAMP), binds to the promoter and stimulates

the interaction of the RNA polymerase with the promoter [83, 114].

The CAP-cAMP complex has been shown to recognise a symmetrical sequence between -55 and -68 bp from the transcription initiation site. RNA polymerase has been shown to interact at two sites, one at between -38 and -27 and the other between -12 and -6 bp from the transcription initiation site. The region -38 to -7 has some similarity to most promoters and evidence suggests it is important in the recognition of the promoter by RNA polymerase. The 7bp region from -12 to -6bp in the *lac* promoter is similar to the sequence TATRATR or Pribnow Box which is present with slight variations in most promoters and is believed to be the RNA polymerase attachment site [46]. P2 is the major *in vitro* binding site for RNA polymerase in the absence of CAP [114].

1.4.2 The *lac* Operator

The operator (O_1) was originally identified through binding and nuclease protection studies with the *lac* repressor [102]. The length of the repressor protected operator fragment is approximately 27bp. Repressor operator crosslinking experiments by Ogata and Gilbert [87] indicate that the repressor contacts the thymine at +1bp with respect to the P1 mRNA start site.

Additional *lac* repressor binding sites, pseudo-operators, have subsequently been found , namely O_2 and O_3 [49]. O_2 is positioned 410bp downstream and

O_3 92bp upstream of O_1. The relative contribution of each operator has been examined *in vivo* and it has been demonstrated that cooperativity between the three *lac* operators, mediated through the repressor is essential for repression of the *lac* operon. Destruction of O_1 results in almost total loss of repression. Destruction of O_2 and O_3 independently results in only a small decrease in repression, however the combined loss of O_2 and O_3 results in 70-fold reduction [86, 71].

1.4.3 The *LacZ* Gene and Product

The structural gene, *lacZ*, is directly adjacent to the promoter operator sequences and the mRNA start site overlaps the operator site [74]. The *lacZ* gene is 3075bp in length, coding for a protein of 1025 amino acids. The encoded protein forms β-galactosidase which is a tetramer of four identical subunits with a native molecular weight of 490kDa. The enzyme is specific for β-D-galactopyranosides, cleaving the anomeric carbon (C1) from the glycosyl oxygen [53, 52] (Figure 2 [105]). The aglycone group can vary widely, from another sugar to an aryl or an alkyl group. In addition to C-O bonds, the enzyme also cleaves C-F and C-N bonds.

β-galactosidase is the only enzyme specifically required for lactose utilisation. The enzyme acts as a transferase as well as a hydrolase. The galactosyl moiety can be transferred to monosaccharides, oligosaccharides, alkyl alcohols

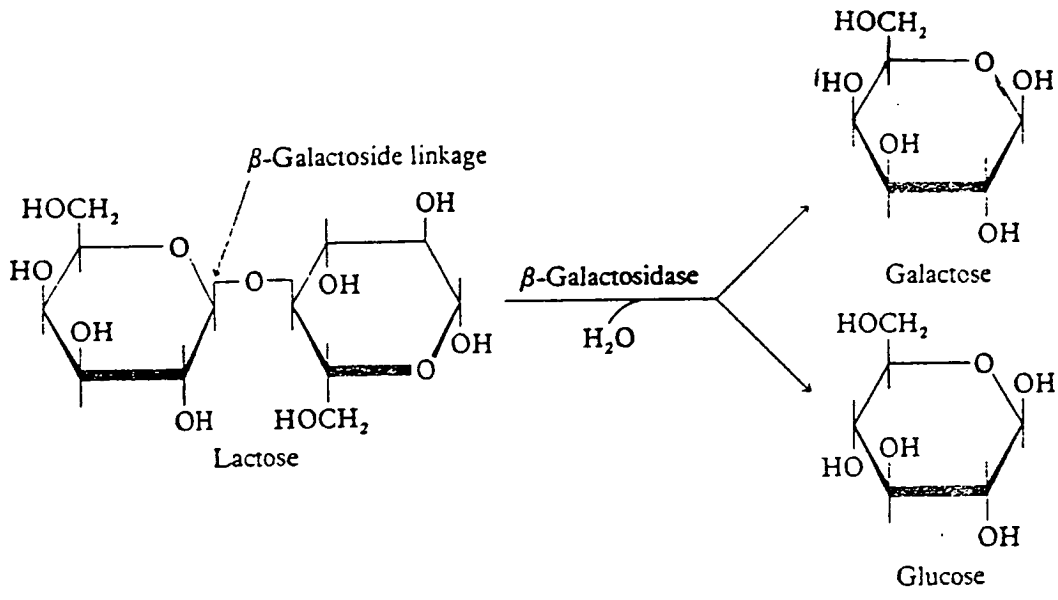
and phenols. It has been shown that allolactose, the natural inducer of the lactose operon, is formed via the transferase activity of β-galactosidase. The galactosyl moiety is transferred from the 4 position to the 6 position of glucose to form allolactose. It has also been shown that allolactose is a better substrate for β-galactosidase than lactose [109, 53] (1975). The K_m's of lactose and allolactose are 5.5 x $10^{-}3$ and 6.3 x $10^{-}4$ respectively.

The enzyme activity can be easily measured with chromogenic substrates such as o-nitrophenyl-β-D-galactopyranoside [54]. This compound is colourless, but in the presence of β-galactosidase it is converted to galactose and o-nitrophenol. The o-nitrophenol is yellow and can be measured by its absorption at OD_{420} as described in the methods Section 3.23.

Figure 2: A Schematic Representation of the Enzyme Reaction of β-galactosidase

The β-galactoside linkage of lactose is cleaved at C1 and the glycosyl oxygen to generate glucose and galactose.

Figure 2: A Schematic Representation of the Enzyme Reaction of β-galactosidase



1.4.4 The *LacY* Gene and Product

The structural gene, *lacY*, positioned 52bp downstream of the *lacZ* gene, is 1254 bp in length and codes for a protein of 418 amino acids. The *lac* permease is a monomer with a molecular weight of approximately 46kDa [91, 113, 9]. Transport and accumulation of lactose in *E.coli* is dependent solely on the *lac* permease [92, 56].

The permease is a hydrophobic protein found in the cytoplasmic membrane [90]. The protein has been solubilised from the membrane, purified to homogeneity and its activity has been reconstituted in phospholipid vesicles [56].

The protein catalyses the coupled translocation of a single β-galactoside with a single H^+ [56, 113]. Under physiological conditions in which the H^+ electrochemical gradient across the cytoplasmic membrane is interior-negative and alkaline, the *lac* permease utilises the free energy released from the downhill translocation of H^+ to drive accumulation of β-galactosides against a concentration gradient. Under the opposite conditions where the electrochemical gradient is interior-positive and acidic, the *lac* permease utilises the free energy generated from the downhill translocation of β-galactosides to drive the uphill translocation of H^+ [75, 56].

Various experimental strategies have been used to examine the mechanism of translocation, in particular site directed mutagenesis has identified specific

residues within the protein which are of importance [77].

A wide range of galactosides have been used in the study of the *lac* permease e.g. dansyl galactosides [56, 53]

There is no simple method of assaying for enzyme activity. However permease activity can be calculated by measuring the accumulation of β-D galactopyranosides against a concentration gradient. Gratuitous substrates, labelled radioactively in most cases, which cannot be metabolised by β-galactosidase are widely used e.g. TMG , IPTG [54], Accumulation of non-metabolised substrates is much easier to measure accurately than metabolisable substrates.

1.4.5 The *LacA* Gene and Product

The structural gene, *lacA*, positioned 64bp downstream of the *lacY* gene, has a coding sequence 609 bp in length, and codes for a protein of 203 amino acids [39]. Strains carrying deletions of *lacA* are able to transport and metabolise lactose normally, therefore the *lacA* product is not necessary for the normal function of the lactose system [2] and therefore will not be discussed further.

1.4.6 The *lac* operon terminators

Examination of the 3′ end of the *lac* mRNA in the region downstream of the *lacA* gene shows several possible endpoints of transcription. Three potential hairpin structures have been identified which may be involved in termination

of transcription, in particular a hairpin 103bp from the end of *lacA*[39].

1.4.7 The *LacI* Gene and Product

The structural gene *lacI* coding sequence is 1083bp in length and codes for a protein of 361 amino acids. The end of the repressor gene is situated just 16bp upstream of the *lac* operator site. Functional repressor is a tetramer with a native size of approximately 152kDa [32].

The repressor protein is present within the cell at all times in low levels. The intracellular concentration is thought to be approximately 10^{-8}M, which corresponds to 10 to 20 molecules per cell.

1.5 Regulation of Operon Expression

Wild type *E.coli* cells grown in the absence of a galactoside e.g. in glucose, contain 1 to 10 units of galactosidase per mg dry weight or 0.5 to 5 active molecules per cell [52, 51, 53]. Cells grown in the presence of a suitable inducer, on the other hand, contain an average of 10,000 units per mg dry weight. This induction effect is brought about by two main mechanisms of transcriptional regulation.

1.5.1 Negative Regulation of Gene Expression

When lactose is absent from the medium, the repressor protein binds to the operator sequences upstream of the three structural genes (Figure3) [52, 51, 53].

Binding of the repressor to these sequences is traditionally believed to result in a steric block to the RNA polymerase, consequently preventing transcription. However recent evidence suggests that the repressor does not prevent RNA polymerase binding but increases the binding 100 fold [53, 71]. The data suggests that the repressor acts by blocking the isomerisation step in transcription initiation. It is also suggested that the repressor holds the RNA polymerase molecule on the DNA until "required" for transcription.

In wild type cells repression of approximately 1000 fold can be observed, due mainly to the interaction of the repressor to the *lac* operator (O_1). The cooperativity of O_1 and one of the other pseudo-operators ensures efficient repression [86, 71].

When lactose is present in the medium it binds to the repressor protein, changing its conformation such that it can no longer bind to the operator sequences [53, 71]. Transcription of the structural genes can then proceed.

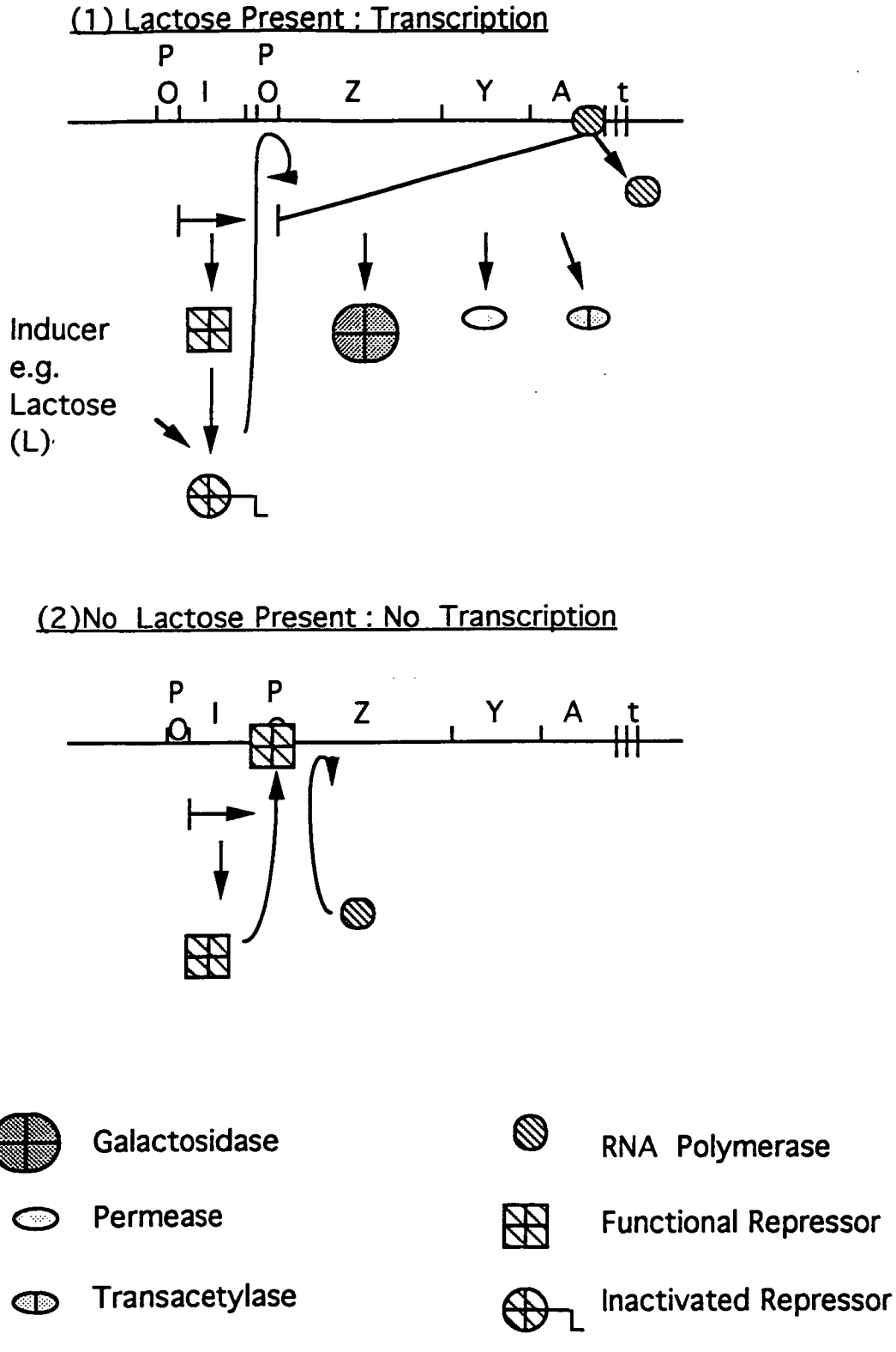
Artificial inducers e.g. isopropyl-thio-β-D-galactopyranoside (IPTG) can also bind to the repressor protein and induce expression. IPTG has been shown to bind the repressor more efficiently than lactose itself [53]. Allolactose has a much higher affinity for repressor protein than either lactose or IPTG [53]. Lactose, on entering the cell, is converted to allolactose by basal levels of β-galactosidase. The allolactose then binds to the repressor protein with greater affinity than lactose, enhancing the induction effect.

Figure 3: Factors Affecting *lac* Operon Expression I

(1)**Lactose Present**; Lactose binds to the repressor inducing a conformational change such that the repressor can no longer bind to the promoter/ operator and transcription occurs.

(2)**No Lactose Present**; The repressor binds to the promoter/operator and prevents RNA polymerase from initiating transcription.

Figure 3 :: Factors affecting *lac* operon expression



1.5.2 Positive Regulation of Expression

As well as the negative control exerted on the operon described above, the operon has a positive mechanism of regulation (Figure 4 i.e. when it interacts with the Catabolite Activator Protein (CAP), or the cyclic-AMP Receptor Protein (CRP) operon transcription is enhanced [114].

When glucose levels are low the CAP protein binds to a controlling element near the *lac* promoter, thereby enhancing transcription. A rise in glucose causes CAP to leave its controlling element and transcription of the operon soon drops to a very low level. Thus the presence of glucose inhibits the synthesis of enzymes involved with the utilisation of sugars such as lactose. Under certain conditions, therefore, even though lactose or another inducer is present the inducibility of the lactose operon is reduced or abolished. This phenomenon, Catabolite Repression or the Glucose Effect, occurs in many micro-organisms including yeast. In *E.coli* the glucose effect is mediated cyclic-AMP (cAMP) and CAP. CAP is sensitive to intracellular levels of cAMP, as long as levels are high CAP stimulates the expression of such operons as *lac*. The introduction of glucose causes a fall in intracellular cAMP levels and therefore a reduction in CAP activity. As a result transcription of CAP activated operons drops to low levels.

This control allows the preferential catabolism of glucose whenever it becomes available. This may be advantageous since, energetically, glucose is the

optimum carbon source.

The *lac* operon is often described as an elegant mechanism by which the cell conserves its energy and resources by producing the lactose metabolising enzymes only when they are "needed" and "useful". Yet the physiological consequences of this type of regulation have not been examined until recently.

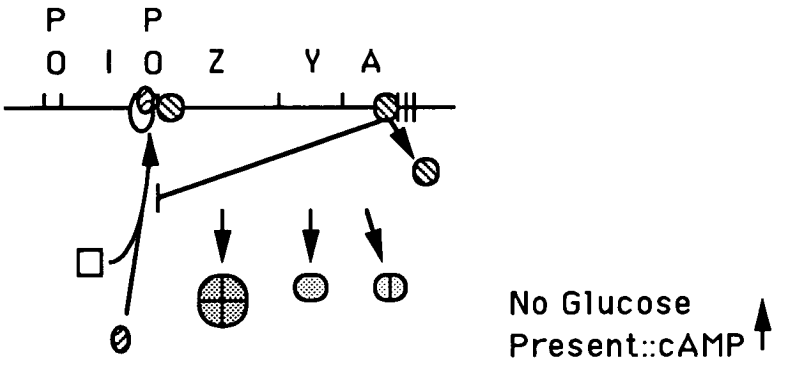
Figure 4: Factors affecting *lac* operon expression II

(1)**No Glucose Present**; In the absence of glucose cAMP levels within the cell are high. cAMP binds to CAP and this complex interacts with the promoter/operator and RNA polymerase to enhance transcription.

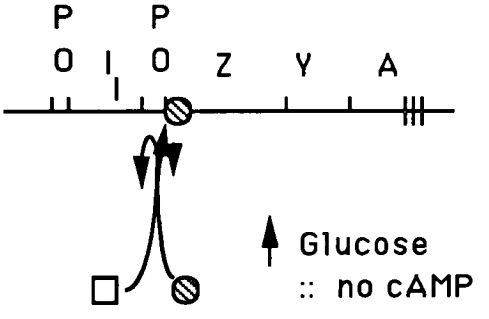
(2)**Glucose Present**; In the presence of glucose cAMP levels are low. In the absence of cAMP CAP is unable to bind to the promoter/ operator.

Figure 4 :: Factors affecting *lac* operon expression

(1)No Glucose Present: No Catabolite Repression



(2)Glucose Present: Catabolite Repression



Galactosidase

Permease

Transacetylase

RNA Polymerase

Catabolite Repressor Protein

cAMP

Activated CAP complex

1.6 Physiological Studies of the *lac* operon

Dykhuizen, Dean and Hartl [30, 29, 24] applied MCA to the *lac* system in *E.coli*. Growth rates/fitness of different strains of *E.coli* were measured in a chemostat, with limiting lactose, and the relationship between fitness and enzyme activity was examined.

1.6.1 Growth Rate/Fitness in a Chemostat

The chemostat is designed to hold a population of bacteria at a constant specific growth rate (μ), lower than the maximum specific growth rate, over many generations (Figure 5) [16, 42, 47, 29]. The population size (biomass) of the bacteria (x) and the volume of the culture (v) is held constant by continuously removing some of the culture medium and cells and replacing it with an equal amount of fresh medium at the same rate, the flow rate(f). The culture is continuously stirred to approximate complete mixing such that the fresh medium entering the chemostat is immediately and uniformly dispersed as well as ensuring a good oxygen supply. One constituent of the fresh medium implicitely required for cell growth, is supplied at a deliberately low level such that its concentration (S_r) in the fresh medium is the sole growth limiting factor. The limiting nutrient therefore prevents the culture from attaining the maximum specific growth rate.

Immediately after inoculation, the cells grow at the maximum specific

growth rate until nearly all of the limiting nutrient has been consumed. The growth rate then decreases until an equilibrium/steady state is reached where the increase in the number of bacteria (μ.x) is equivalent to the number siphoned off with the old medium (D.x), where D is the dilution rate (f/v). At steady state the change in the population size of the culture (dx/dt) is zero, therefore;

$$increase \ = growth \ - output \ = 0 \tag{2}$$

or

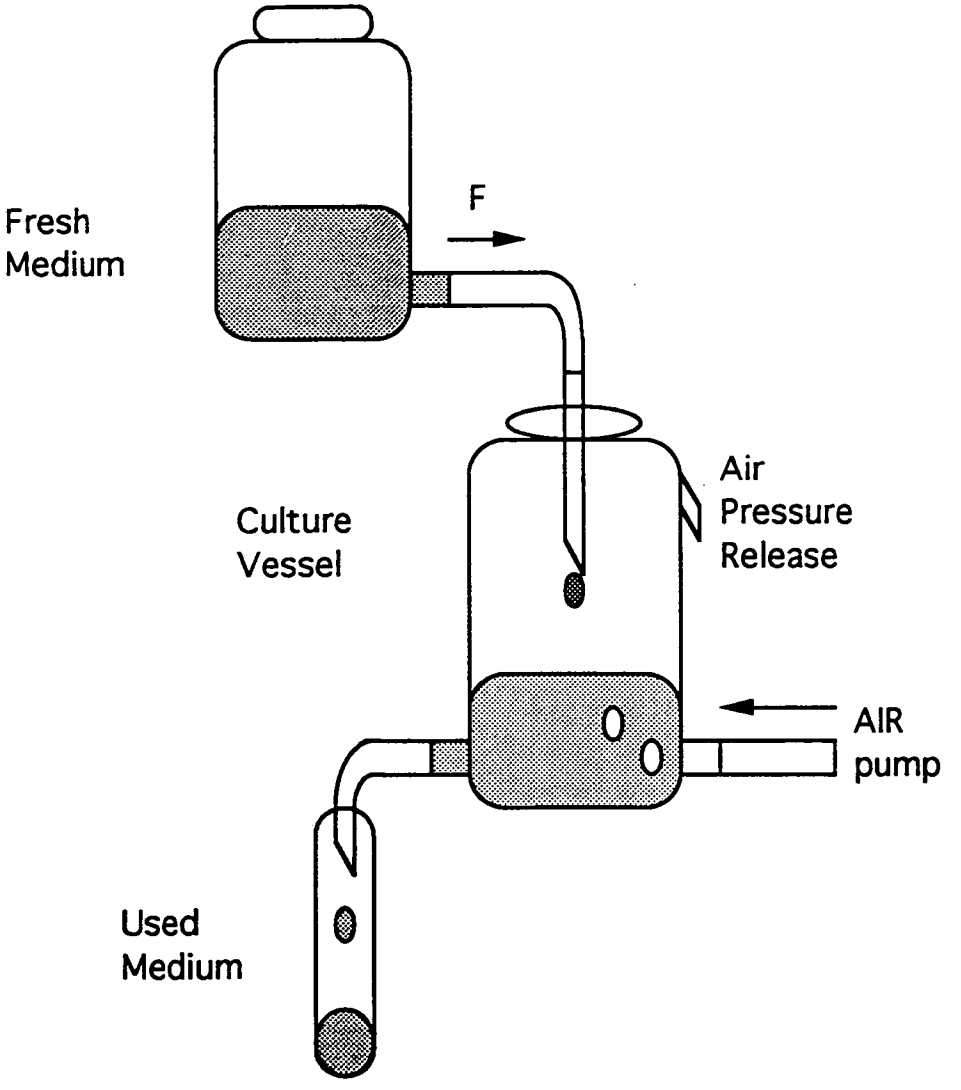
$$\frac{dx}{dt} \ = \mu x \ - Dx \ = 0 \tag{3}$$

Thus, as the overall density or population of cells in the chemostat vessel is constant once the steady state is reached, the growth rate equals the dilution rate which is set by the experimenter. Measurement of x, the cell density at steady state therefore gives μ, the specific growth rate of the strain.

Figure 5: A Simple Diagram of a Chemostat

Fresh medium enters the chemostat at a flow rate (F) and is immediately and uniformly dispersed in the chemostat culture vessel. Used medium is removed at the same flow rate into a collection or sampling vessel.

Figure 5 :: A Simple Diagram of a Chemostat



1.6.2 Variation of Substrate concentration

In the chemostat the limiting nutrient is supplied in the incoming medium, at a concentration (S_r) diluted in the culture vessel, consumed by the cells for growth, and removed from the culture medium at a concentration (S) [95, 42, 29]. The relationship between a limiting substrate and growth may be written as;

$$\mu = V[S]/(K_s + [S]) \tag{4}$$

where [S] is the substrate concentration of the limiting nutrient in the chemostat, V is the maximum specific growth rate [42, 29] and K_s is a constant and implies a hyperbolic relationship [95]. Although the concentration of the bacterial culture (biomass) is dependent on S_r (the concentration of limiting nutrient in the fresh medium) the growth rate is not. The concentration of substrate in the chemostat is independent of S_r i.e. when the dilution rate is fixed the substrate concentration must come to a level such that μ is equal to D and this level is independent of S_r.

Thus varying substrate concentration (S_r), while maintaining the dilution rate, results in variation of the biomass, i.e. the population size of the culture only, and the growth rate remains constant.

1.6.3 Competition In Chemostat cultures

The population size, at steady state, is determined by the supply of the limiting nutrient in the fresh medium (S_r) entering the chemostat, but the growth rate of the population of cells is determined by the concentration of the limiting factor in the chemostat vessel itself [S] i.e. the concentration in the immediate environment of the cells and parameters of the strain itself e.g. the maximum specific growth rate [73, 29]. Cell density and the doubling time are therefore independent.

Competition experiments for the limiting nutrient between different strains of bacteria are therefore possible. In a competition experiment between two strains, 1 and 2, the change in total population size of the culture can be written as;

$$\frac{dx}{dt} = \mu_1 x_1 + \mu_2 x_2 - D(x_1 + x_2) \tag{5}$$

where the change in the population size of the culture is dx/dt and μ_1 is the growth rate of strain 1 and μ_2 is the growth rate of strain 2. The individual growth rates are strain specific and dependent on [S] (Equation 7), the concentration of the limiting nutrient, common to both strains, in the chemostat vessel and the growth parameters of the respective strains.

At "equilibrium" dx/dt is zero, therefore

$$D = \frac{\mu_1 x_1 + \mu_2 x_2}{x_1 + x_2} \tag{6}$$

and the weighted sum of the growth rates of both strains is equal to the dilution rate but the growth rate of the individual strains can vary. The strain which can utilise the limiting nutrient more efficiently will have a faster growth rate and will contribute a greater proportion of offspring to the next generation. Since the total population size is constant, the strain with the faster growth rate will outgrow the poorer growing cells and eventually take over the entire population. If the proportion of each strain is monitored through several generations of growth, by following a neutral marker, a measure of the relative growth rate, which in this case may be considered as a measure of relative fitness, can be estimated. Thus the behaviour of different strains compared to a control strain can be examined.

1.6.4 Experiments Prior to this Work: Variation in *lacZ*

Dykhuizen *et al* [30, 24, 23] initially produced a series of *lacZ* mutants covering a wide range of activities. The parent strain CSH10 [54] containing a nonsense mutation within the *lacZ* gene, was unable to grow on lactose. This strain was used to produce revertants, or missense mutations, by selecting for growth on lactose. A second class of β-galactosidase activities, encoded by the *ebgA* gene (evolved β-galactosidase), comprising less than 0.5% of the fully induced wild-type activity was isolated from a *lacZ* deletion strain. Pre-existing mutants of *lacZ* were also used in the analysis. The wild-type *lac* operon of CSH64

and constitutive operon of JL3300 were used as controls [30, 29]. It should be noted that the permease in all the mutants was unaffected.

The mutant and control operons were transduced into strain DD320 to provide a uniform genetic background. Competition experiments between pairs of strains were then carried out in a chemostat in which the limiting nutrient was lactose. When strains of *E.coli* are placed in competition for limiting concentrations of lactose, the flux from lactose limits the rate of growth and therefore growth rate/fitness would be expected to be a function of this flux. Resistance to the bacteriophage T5, induced in one of the competing strains (and the reciprocal experiment), was used as marker and the proportion of each strain was monitored as they competed for limiting lactose.

The ability of the different strains to utilise lactose and grow was taken as a measure of their relative fitness.

The data obtained from these studies demonstrated that growth rate/fitness was related to enzyme activity in a non-linear hyperbolic manner. This is consistent with the control analysis when systems with low saturation of the enzymes occur [60]. From the set of data (Figure 6) [24] the control coefficient for the fully induced wild type β-galactosidase activity was estimated to be C=0.018.

Furthermore, such low values were observed over a wide range of β-galactosidase activities, until the exceedingly low enzyme activities were present.

Figure 6: Relative Fitness as a Function of β-galactosidase Activity

The effect on fitness of changing the activity of β-galactosidase while holding permease at a constant value of one. Both fitness and enzyme activity are presented relative to the values for the wild-type-induced level of activity. Obtained from Dykhuizen *et al* 1987 [29].

Figure 7: Relative Fitness as a Function of Permease Activity

The effect on fitness of changing the activity of permease while holding β-galactosidase at a constant value of one. Both fitness and enzyme activity are presented relative to the values for the wild-type-induced level of activity. Obtained from Dykhuizen *et al* 1987 [29].

Figure 6:: Relative Fitness as a Function of B - Galactosidase Activity

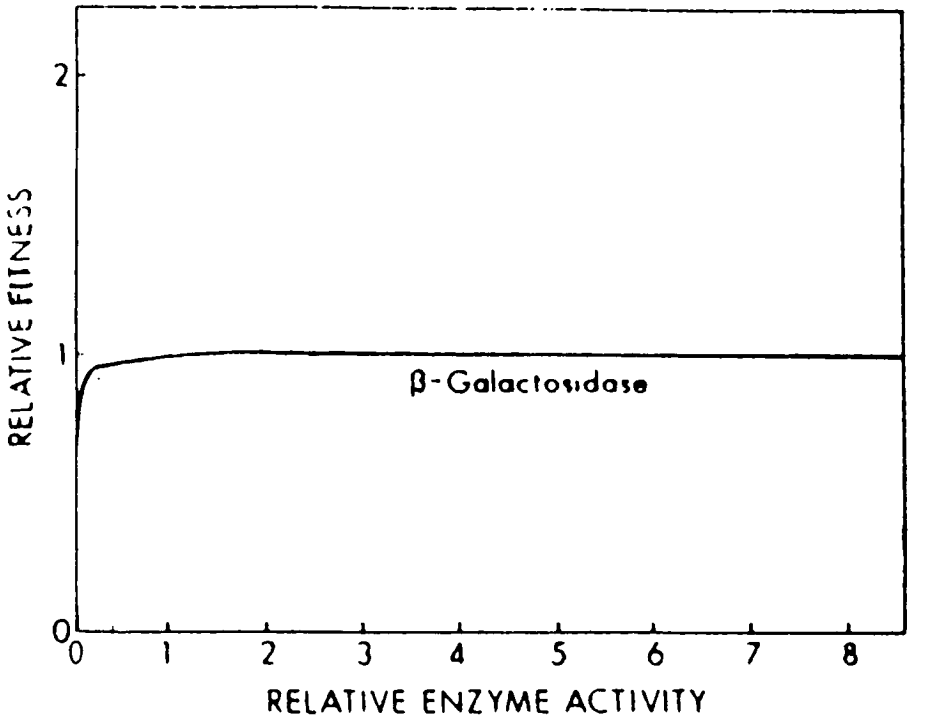
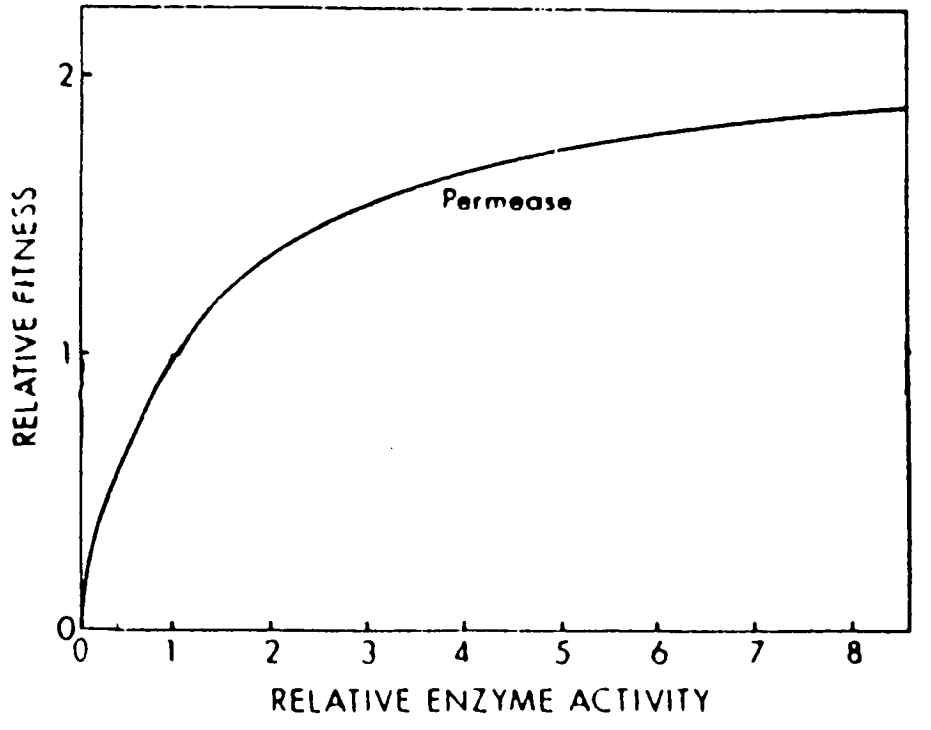


Figure7:: Relative Fitness as a Function of Permease Activity



1.6.5 Variation in *lacY*

Dykhuizen *et al* then extended their analysis to the lactose permease using the wild type *lac* operon in the DD320 background. The wild type operon was induced to varying degrees using IPTG and grown in competition with a *lacI* constitutive strain in a chemostat with limiting lactose. Permease activity was not assayed directly but was calculated from the relative β-galactosidase activities on the assumption that the operon is coordinately induced by IPTG. The results are shown in Figure 7 [24].

The results of the induction experiments plus the previous β-galactosidase experiments were plotted using multiple linear regression to obtain a three dimensional representation of the fitness surface (Figure 8) [29, 24]. The measured control coefficient at wild type fully induced levels for the *lac* permease turned out to be large, C=0.551.

The permease therefore is much more "important" in determining the flux through the pathway at these enzyme levels than β-galactosidase. The coordinate induction of β-galactosidase and permease results in an increased flux principally because of the change in permease, the change in β-galactosidase being almost irrelevant. The authors suggest that, as a consequence of this high value, natural selection would be expected to favour polymorphisms or direct selection in the permease which would increase the efficiency of the enzyme and, thus, reduce its control coefficient. The observed high coefficient

suggests that other forces are acting to maintain the permease efficiency at a possible optimum. The consequences to the cell membrane potential may be a factor. Alternatively, the requirement of being part of the membrane may limit the possible structural improvements.

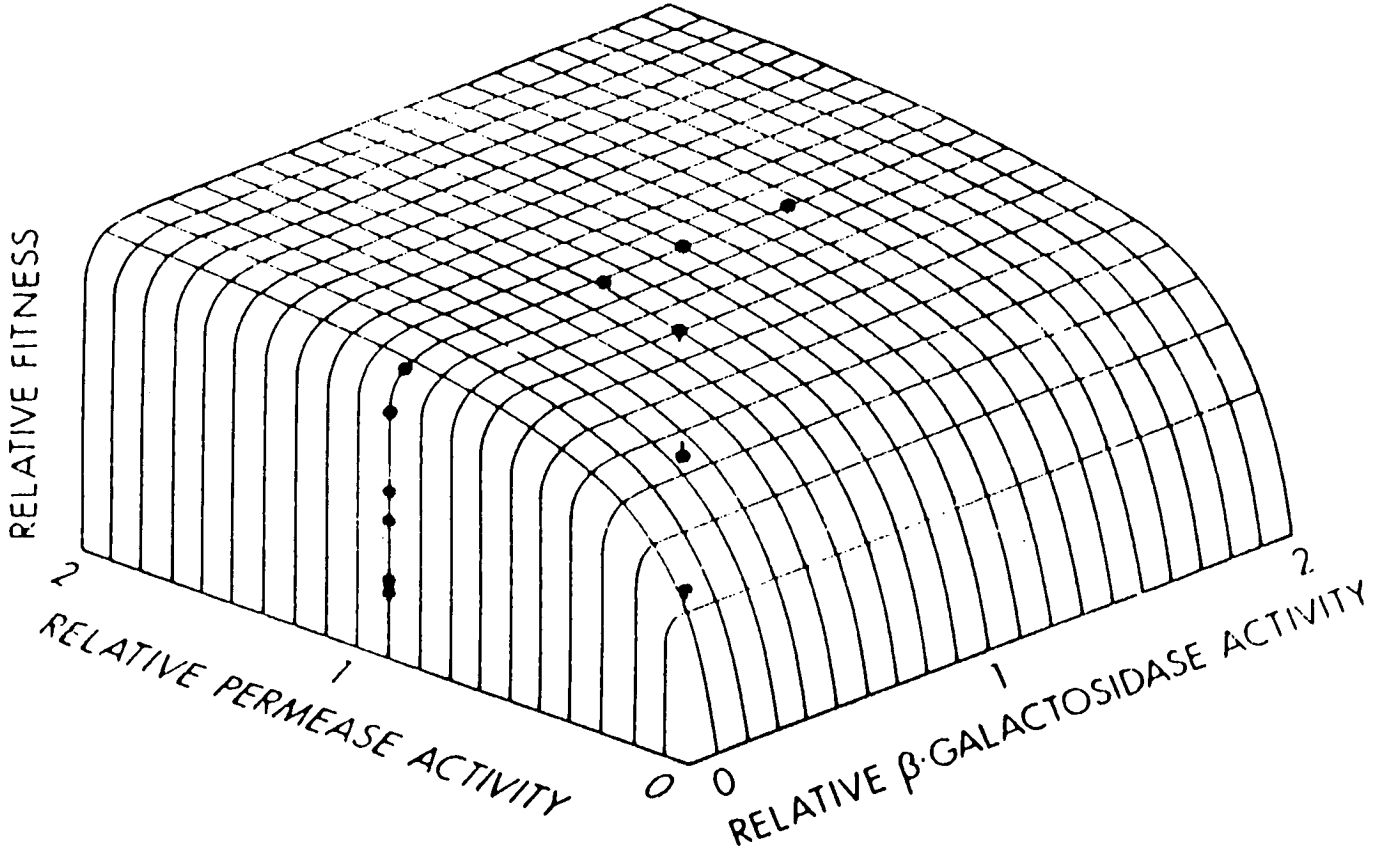
Subsequent to these studies Dean examined the transport of galactosides further, by measuring the effect of naturally occuring polymorphisms [23]. Using the same approach as described above the cell wall was shown to impose a resistance to the diffusion of galactosides at low substrate concentrations. The variation in permease activity still had a demonstrably greater effect on fitness than the variation in β-galactosidase, although not as great an effect as expected from the previous work.

Figure 8: 3-Dimensional Control Surface for the *lac* Operon

Three dimensional representation of the fitness surface. Lines are placed at 0.1 intervals of enzyme activity, relative to a value of one for the constitutive level of expression. The dots are the data points used in estimation of the parameters. Obtained from Dykhuizen *et al* 1987 [29].

Figure 8:: 3D Fitness Curve

D. E. Dykhuizen, A. M. Dean and D. L. Hartl



1.7 Aim of Thesis and Experimental Strategy

The aim of this thesis was to extend the physiological analysis of the operon, using a different strategy which it was hoped would bypass some of the problems of the previous study. The number of points which generated Figure 8 are rather few, particularly for permease over the region where rapid changes in control coefficient take place. This is important in revealing the epistatic relationship between the two enzymes.

One drawback of the previous study was the use of specific mutants which generated data only for each discrete enzyme activity. Ideally, a more complete data set would be desirable covering as wide a range as possible. More importantly, the use of different mutations with different enzyme activities may have resulted in changes in the parameters of the enzyme e.g. K_m, Vmax, such that a number of parameters are different simultaneously in each case. Changing only the enzyme concentration and holding everything else constant would generate more reliable and interpretable results.

The ideal system for studying the operon using metabolic control analysis was thought to be to split the operon up such that the *lac* genes, *Z* and *Y*, are under the control of different inducible promoters. Such a system would allow the independent expression of the operon genes to different extents over a wide range of levels, dictated by the promoters chosen. The same genes would be used throughout the analysis and hence the enzyme parameters would be

constant. It would then be relatively easy to set the levels of the respective enzymes and assess the fitness.

One of the genes could remain under *lac* promoter control. The second promoter would, necessarily, have to be inducible over a sufficiently wide range of activities to compare with the *lac* promoter. Clones of all the operon genes were available to be subcloned as well as a range of cloned promoters.

It was necessary to build into the overall experimental strategy a mechanism to enable stable maintenance of the cloned gene copy number. In chemostat studies plasmid stability is a serious problem. A single stably integrated copy of the gene construct into the *E.coli* chromosome would bypass any problems of stability. Inherent in the strategy, therefore, was the requirement for homologous recombination/ integration with the *E.coli* chromosome.

The transacetylase would essentially be ignored, as in the previous analysis, since it is unclear what its role is, if any, in lactose metabolism and it has no obvious effect on fitness.

The side reactions of β-galactosidase i.e. the production and subsequent breakdown of allolactose have also been ignored in this analysis. Under steady state conditions, the side reactions are at equilibrium such that any lactose converted to allolactose must also be hydrolysed to glucose and galactose. The overall effect on flux of these side reactions is, therefore, thought to be negligible.

The desired endproduct was a strain containing single copies of the *lacZ* and *Y* genes, stably integrated into the chromosome such that they can be induced independently of one another and to varying extents. In fact, the permease gene was detached from *lac* promoter control and placed under the control of the *trp* promoter. A wide range of enzyme activities can then be analysed, in chemostat experiments on limiting lactose, to expand and fill in the gaps in the data as it exists to date.

In regions of steeply rising flux values for modulations of either enzyme, the control coefficient changes (as indicated by the change of slope). When such a change has taken place, the value of the control coefficient of the other enzyme will necessarily have changed because of the operation of the Summation Theorem. Thus, for the case, reported by Dykhuizen *et al*, we have

$$C^J_{perm} + C^J_{\beta-gal} + \sum C^J_{E_i} = 1 \qquad 0.551 + 0.018 + 0.431 = 1 \qquad (7)$$

where $\sum C_{Ei}$ is the sum of the other coefficients.

If the permease activity is increased by a given amount by the induction, the coefficient will decrease to, say 0.3, the sum of the [$C^J_{\beta-gal}$ + $C^J_{E_i}$] will increase to 0.7. At the new point the $C^J_{\beta-gal}$ is expected to have risen to 0.028. With a greater permease increase, to a lower coefficient of, say 0.1, the $C^J_{\beta-gal}$ value will have risen to 0.036, i.e. it will have doubled its sensitivity to affect flux. This type of analysis is only possible by the method of independent induction of the two enzymes.

Further to this it may be possible to use this system to examine questions relating to the selective forces that maintain the lactose operon in a regulated state in natural populations.

In the event time was too short to make many physiological experiments, the majority of time having been spent in the construction of strains. An unfortunate blind alley in attempting to integrate the *lac* construct consumed a large part of the time. Nevertheless the strain is now constructed and has been shown to respond to the different inducers in the expected fashion.

2 Materials

2.1 Chemicals

A list of the suppliers of chemicals is given in Table 1.

Table 1: Chemicals

Chemical	Supplier
General Chemicals	BDH, Aldrich.
Media	Difco.
General Biochemicals	Sigma, Pharmacia, BRL, Boehringer Mannheim.
Agarose	Sigma.
Acrylamide	Sigma.
NN'methylene bisacrylamide	Sigma.
Radiochemicals	Amersham.
Restriction Enzymes	Boeringher Mannheim, Promega.
Restriction Buffers	Boeringher Mannheim, Promega.
Other Enzymes	Boeringher Mannheim, Promega.
Enzyme buffers	Boeringher Mannheim, Promega.
IAA	Aldrich.
Nitrocefin	Glaxo
In vitro Packaging Kit	Amersham
Sequenase Kit	USB.

2.2 *E.coli* strains

A list of *E.coli* strains used and created in this study is given in Tables 2 and 3.

Table 2: *E.coli* Strains

Strain	Genotype	Source
NM522	Δ(*lac-proAB*), *supE*, *thi*$^-$, *hsd*Δ5, F′ *proAB*,*lacI*^{q}*Z*Δm15	N.E.Murray
W3110 (PBN37)	*amp*^{r}, *thyA*36,*deoC*2,*IN*1, F$^-$, λ^-	B.P.Nichols
6947	*lacZ*$_o$15, *relA*1, *spoT*1, *thi*$^-$, λ^-	B.Bachman
6378	*lacI*694, *relA*1, *spoT*1, *thi*$^-$, λ^-	B.Bachman
NE4	DP90CNal background, Δ(*lac-pro*), *thi*$^-$, *nalA*	H.Bocklage
MAA13	DP90CNal background, Δ(*lac-pro*), *thi*$^-$, *nalA*	H.Bocklage
MAA13 (pGB2601)	DP90CNal background, Δ(*lac-pro*),*thi*$^-$, *nalA*, *recA*, F′ *lacI*^{q} *Y*$^-$, MUB7*pro*$^+$	H.Bocklage
RE478	CA7027 background, *lac*Δ46	K.Forbes
RE484	CA7027 backround, *lac*$^-$	K.Forbes
DD320	Δ*lac*RV	J.Langridge
G11	*lacI*$^+$, *Z*$^+$, Δ *Y*, *proC*$^-$	J.Langridge
NM621	*leu*$^-$, *proB*1, *thy*$^+$, *recD*(AC301), *supE*, *tonA*, *hsdR*, *mcrA*, *mcrB*	N.E.Murray
C600	*leu*$^-$, *thr*$^-$, *thi*$^-$, *supE*,*tonA*,*lacY*	N.E.Murray
NM539	P2 lysogen, *supF*, *hsdR*, *lacY*	N.E.Murray

Table 3: *E.coli* Strains continued

Strain	Genotype	Source
NM611	*supF*, *supE*, *recD*, *recBC*	N.E.Murray
NM531	*recA*$^-$, *supF*, *supE*, *hsdR*, *trpR*, *lacY*	N.E.Murray
NM311	*trpA*$^-$, *sup*$^\circ$	N.E.Murray
NM127	*trpB*$^-$, *sup*$^\circ$, *tonA*	N.E.Murray
NM312	*trpC*$^-$	N.E.Murray
NM675	*supE*	N.E.Murray
NM546	*ara*$^-$, *thi*$^-$, Δ(*lac-proA*), F$^-$	N.E.Murray
DL615	$sbcC_{201}$,*proC*29, *phoR*79::Tn10(tet), *sbcB*15, *his*4, *arg*3, *recB*21, *recC*22 *galK*2, *mtl*1, *xyl*5, *supE*44, *rsp*31	D.Leach
NM614	Prototrophic, λ^-, r^-, m^+	N.E.Murray
614*lac*ΔY	NM614 carrying *lac*ΔY from G11	Chapter5
614(5)	614*lac*ΔY carrying lysogen of phage 1	Chapter5
614(5)*trp*$^-$	614(5) cured derivative, with insertion of *lacY* construct into *trpB* region to generate Trp$^-$ phenotype	Chapter5
614(5)*trp*$^+$	614(5) cured derivative, with loss of insertion of *lacY* construct from the *trpB* region	Chapter5
614(14)	614*lac*ΔY carrying lysogen of phage 2	Chapter5
614(14)*trp*$^-$	614(14) cured derivative, with insertion of *lacY* construct into *trpB* region to generate Trp$^-$ phenotype	Chapter5
614(14)*trp*$^+$	614(14) cured derivative, with loss of insertion of *lacY* construct from the *trpB* region	Chapter5

2.3 Plasmids

A list of the plasmids used and created in this study is given in Table 4.

Table 4: Plasmids

Plasmid	Description	Source
pGB2601	*ColE1* plasmid carrying *lacY* subclone (2.1kb)	H. Bocklage
PBN37	pBR322 based plasmid carrying *S.marsescens trp* promoter (0.25kb)	B. P. Nichols
PBN371	PBN37 with downstream EcoRI site (position 1.32kb) destroyed	Chapter3
PBN372	PBN37 with upstream EcoRI site (position 1.06kb) destroyed	Chapter3
PBN372y	PBN372 with *lacY* subclone in correct orientation	Chapter3

2.4 Phages

A list of the phages used and created in this study is given in Table 5.

Table 5: Phages

Phage	Description	Source
λNM1224	λb1453, *chi*$^-$	N.E. Murray
λNM1134	λamp^r, *lacZ*, i^{21}, *nin*, h^λ	N.E. Murray
λNM220	λ*c*I26, *h*80, att^λ	N.E. Murray
λNM63	λ*c*I26	N.E. Murray
λNM1	λ*c*I857	N.E. Murray
λNM817	λ*trpABC*$^+$, i^{21}, *nin*	N.E. Murray
λNM1249	EMBL3 *c*I857	N.E. Murray
λ1249*trpABC*	λ1249 containing 9kb fragment of λ817 containing the *trp ABC* genes	Chapter5
λPhage1	λ*c*I857 containing HindIII-SalI fragment of PBN372y	Chapter5
λPhage2	λ1249*trpABC* containing *lacY* construct	Chapter 5

2.5 Media and Solutions

Formulae for the media and solutions used in this study are given in the Appendix.

3 Methods

3.1 Small scale isolation of *E.coli* Genomic DNA

A single colony of *E.coli* cells was used to inoculate 5mls of LB medium and incubated at 37°C overnight with constant shaking.

1ml of this culture was transferred to an Eppendorf and microfuged for 1min. The supernatant was removed from the pellet with aspiration and the pellet resuspended in 500μl TEL and incubated for 30mins in a 37°C water-bath.

0.1ml of SDS/RNAse solution was added and the DNA solution was then phenol extracted 2 times. In the first phenol extraction, the DNA solution was extremely glutinous and it was necessary to mix it with the phenol gently for over an hour before microfuging. It was also necessary to remove a white glutinous pellet formed at the interface before removing the upper aqueous layer. The second phenol extraction was carried out as normal.

The DNA solution was then chloroform extracted 1x and precipitated by mixing well with 15μl of 5M NaCl and 1ml EtOH. The DNA was pelleted by microfuging for 5mins, the pellet was washed in 70% EtOH, dried and resuspended in 100μl TE.

3.2 Isolation of Plasmid and Bacteriophage DNA

3.2.1 Large Scale Isolation of Plasmid DNA

A single colony of *E.coli* cells containing the plasmid of interest was used to inoculate 25mls of LB medium, containing 75μg/ml of the appropriate antibiotic and incubated at 37°C overnight with constant shaking.

12.5mls of this culture was used to inoculate 500mls LB, with 75μg/ml of the appropriate antibiotic, and this was then incubated overnight at 37°C with constant shaking.

The cells were harvested by centrifugation, in a Sorval GSA rotor, at 7K for 10mins at 4°C. The supernatant was removed by aspiration and the pellet resuspended in 8mls (per 500mls of original culture) of Solution I containing lysosyme at final concentration of 5mg/ml, transferred to Polypropylene tubes, and stored on ice for 10mins.

16mls (per 500mls of original culture) of freshly prepared Solution II were added, the tubes mixed by vortexing and placed on ice for 10mins. To precipitate cell debris 12mls (per 500mls of original culture) of Solution II were added, mixed thoroughly and placed on ice for 10mins.

The cell debris was pelleted by centrifugation at 10K for 30mins at 4°C and the supernatant transferred, by filtration through a blue tip plugged with polyallomer wool, into two 30mls Corex tubes (18mls per tube).

10mls of isopropanol were then added to each tube, mixed, and the tubes

were placed at room temperature for 30mins. The DNA was pelleted by centrifugation for 30mins at 10K and room temperature. The pellet was washed in 70% Ethanol, vortexed and centrifuged again for 30mins at 10K and room temperature. The pellet was dried thoroughly and resuspended in 9.5mls TE pH 7.4 (per 500mls original culture), initially by placing at 37°C for 10mins and later by vortexing. 20μl of 10mg/ml RNAse were then added and the tubes placed at 37°C for 20mins.

Exactly 9mls were transferred to a fresh Corex tube and exactly 9.5g of CsCl and 0.5ml of 10mg/ml EtBr were added to give a specific density of 1.55g/ml. The solution was centrifuged at 10K for 10mins to remove any precipitate and transferred with a pasteur pipette to an ultracentrifuge tube (Beckman). When necessary the tubes were topped up with 0.95g/ml CsCl/TE solution. The tubes were then balanced to within 0.05g and centrifuged for 16hrs at 45K and 18°C in a Beckman Ti70 rotor.

The band of DNA was visualised under UV and removed using a wide gauge needle. The EtBr was extracted by adding 2 x volume of isoamyl alcohol, mixing by inversion for 5mins, microfuging for 3mins at room temperature in Eppendorf tubes and discarding the upper layer. The isoamyl extraction was repeated 3-6 times until all the EtBr was removed. The aqueous phase was dialysed against several changes of TE(pH 8.0) or overnight.

The OD_{260} versus OD_{280} was measured of a 1:50 dilution, a value of between

1.8 and 2.0 was required for a clean preparation.

3.2.2 Small Scale Preparation of Plasmid DNA

A single colony of *E.coli* cells, containing the plasmid of interest was used to inoculate 5mls of LB medium, containing 75μg/ml of the appropriate antibiotic and incubated overnight at 37°C with constant shaking.

1.5 mls of this culture was removed to an Eppendorf tube and the cells were recovered by centrifugation in a microfuge for 2mins. The supernatant was removed by aspiration and the cells resuspended in 200μl of Solution I containing lysosyme at a final concentration of 5mg/ml (added immediately prior to use). The contents of the tube were mixed by vortexing and stored at room temperature before adding 200μl of freshly prepared Solution II.

150μl of ice cold Solution III was then added and the tube vortexed in an inverted position gently for 10secs and placed on ice for 5mins. The cell debris was pelleted by centrifugation for 10mins and the pellet discarded. The supernatant was phenol extrated 2x and 1ml of EtOH was added to the supernatant. The tube was left at room temperature for 2mins to precipitate the plasmid DNA and then microfuged for 10mins to pellet the DNA. The supernatant was removed by aspiration and the pellet washed with 1ml of cold 70% EtOH and again the EtOH was removed by aspiration. The pellet was dried and resuspended in 50μl of TE.

3.2.3 Large Scale Isolation of Bacteriophage DNA

A single colony of *E.coli* cells, sensitive to the bacteriophage of interest, was used to inoculate 30mls of LB medium and incubated at 37°C overnight with constant shaking.

3.0mls of this culture were used to inoculate 100mls of LB medium containing 1ml of 1M $MgSO_4$ and the culture was shaken vigorously at 37°C. The OD_{650} was monitored until it reached 0.45 (2x 10^8 bacteria per ml) and bacteriophage were added to a multiplicity of infection of 0.1 (2 x 10^9 phage).

The culture was returned to vigorous shaking at 37°C and the OD_{650} was monitored until it dropped to a constant reading. 200μl of $CHCl_3$ were then added and the culture was shaken for a further 10mins at 37°C.

At this stage the culture was titred (100μl of 10^{-7} dilution per plate) to check there was sufficient phage to continue.

The culture was then centrifuged for 10mins at 4K and 4°C and the supernatant poured into another centrifuge bottle. 4g of NaCl and 10μl of a 10mg/ml solution of DNAse and RNAse were then added and placed at room temperature for 1 hr. 10g of PEG 6,000 were then added, swirled gently to dissolve and left for 1 hr or overnight at 4°C to precipitate the bacteriophage.

If the previous day's titre exceeded 5 x 10^9 phage per ml then the culture was remixed and the bacteriophage was precipitated by centrifugation for 10mins at 10K in a pre-chilled rotor. The supernatant was decanted and any

remaining supernatant was soaked up with a tissue.

6mls of SM phage buffer was added and the bottles were slanted on ice for 2 to 3 hrs with occasional gentle swirling to resuspend the phage pellet and avoid the production of any bubbles.

Once the pellets had dissolved an equal volume of chloroform was added, then vortexed and centrifuged for 15 mins at 4K and room temperature. The supernatant was transferred to sterile vials on ice.

A CsCl step gradient was prepared using a drawn out Pasteur pipette with 1.5mls of 31%, 45%, 56% solutions of CsCl (w/v) in SM phage buffer. The sample was overlayed onto the gradient carefully and the tube filled to 3-4mm from the top and balanced with another tube to within 0.05g with SM.

The sample was then centrifuged for 1.5-2 hrs at 33K and 20°C and the discrete blue opaque was removed using a 1ml syringe and 25G needle. The needle was used to bore a hole into the side of the tube to remove the band.

If the band was clean and sharp it was dialysed but if it was mixed with the cell debris band then it was necessary to put it through an equilibrium gradient. The band was added to a 5ml tube with 41.5% CsCl solution, mixed and centrifuged at 33K for 24hrs. The opaque discrete blue band was removed using a 1ml syringe and a 25G needle.

The band was dialysed for a few hours with a change of buffer (TE for DNA: SM for phage) and split into two Eppendorfs. An equal volume of Tris

equilibrated phenol was added, the tubes were mixed gently by inversion and microfuged for 2mins at 10K. The bottom layer of phenol was removed with a drawn out pasteur pipette and the phenol extraction was repeated a further 2 to 3 times. The remaining solution was dialysed overnight to remove any phenol.

The DNA concentration was measured and the solution was EtOH precipitated to concentrate the DNA if necessary with 1/10 volume 3M NaAc and 2.5 x volume of EtOH and placed at -70°C for 30mins. The DNA was then precipitated by centrifugation for 20mins at 10K and 4°C. The DNA pellet was dried in a freeze drier and resuspended in 200μl of TE.

The OD_{260} versus OD_{280} was measured to estimate the concentration of DNA.

3.2.4 Small Scale Isolation of Bacteriophage DNA

A single colony of *E.coli* cells, sensitive to the bacteriophage of interest, was used to inoculate 5mls of LB medium and incubated overnight at 37°C with constant shaking.

100μl of this culture were used to inoculate 4mls of LB medium containing 400μl of 100mM $MgSO_4$ in a sterile universal bottle. Following this $1x10^8$ phage were added and the culture was incubated at 37°C with constant shaking until lysis occurred (3-5 hrs).

A few drops of $CHCl_3$ were added and the culture was vortexed. The cell debris was pelleted by centrifugation in a bench centrifuge for 10 mins at room temperature. A sterile glass 1ml pipette was used to transfer the supernatant to a fresh universal bottle and 5μl of 10mg/ml RNAse and DNAse solution were added.

The solution was incubated for 30mins with constant shaking at 37°C before being transferred to a sterile 15ml Corex tube containing 4mls PEG solution. The solution was mixed thoroughly but gently and placed at 4°C overnight (or 1 hr), then the phage was precipitated by centrifugation for 20mins at 10K and 4°C.

The supernatant was removed by aspiration and the tube was placed in an inverted position on a tissue to drain excess PEG. A small piece of tissue was used to remove any remaining PEG solution. The phage pellet was resuspended gently in 0.5ml of SM phage buffer, 0.5ml of $CHCl_3$ was added, and the solution was vortexed in three brief bursts of a few seconds.

The solution was transferred to an Eppendorf and spun in a microfuge at 5K for 3mins. The supernatant was transferred to a fresh Eppendorf, 500μl Tris equilibrated phenol and 100μl TE were added and the tube mixed by inversion. The Eppendorf was then spun in a microfuge at 10K for 2mins and 500μl of the supernatant were transferred to a fresh Eppendorf. 500μl of Phenol: chloroform (1:1) were added, the tube mixed gently by inversion

and spun in a microfuge at 10K for 2mins. 450μl of the supernatant were transferred to a fresh Eppendorf and 500μl of $CHCl_3$ were added. The tube was mixed by inversion, spun in a microfuge for 2mins at 10K, and 400μl were transferred to a fresh Eppendorf.

800μl of EtOH, at room temperature, were added and placed on ice for 10mins to precipitate the DNA. The DNA was pelleted by centrifugation in a microfuge for 10mins at 10K and room temperature. The EtOH was poured off and the pellet was washed in 70% EtOH, this was removed using a drawn out pasteur pipette and the pellet resuspended in 400μl TE.

The pellet was resuspended gently, then the DNA was precipitated with 40μl 3M NaAc and 800μl of EtOH and placed on ice for 10mins. The DNA was pelleted by centrifugation in a microfuge for 10mins at 10K and room temperature. The EtOH was poured off and the pellet was dried for 5mins in a freeze drier. The pellet was resuspended in 50μl TE.

3.3 Manipulation of DNA

3.3.1 Recovery of DNA from Agarose Gels using NA45 paper

The gel was examined under short wave UV illumination and photographed as quickly as possible to prevent damage to the DNA in the gel.

The fragment to be isolated was identified and a small slot was cut with a scalpel on the right hand side of the band. A small piece of NA45 paper

was inserted in the slot, making sure no contamination from other bands could occur.

The band of DNA was then electrophoresed onto the NA45 paper in a minigel rig at 60mA ensuring the gel was straight, to prevent the band missing the paper. Once the band had run into the paper, the paper was removed from the gel and washed three times in 10mM Tris.

The paper was then transferred to an Eppendorf, immersed in DEAE elution buffer (approximately 100μl) and placed in a 60°C water bath for 30mins or until there is no DNA left on the paper. This was checked by examination of the paper under UV.

The NA45 paper was removed and the DNA diluted to 0.5M salt by adding an equal volume of water and microfuged for 2mins to spin everything to the bottom of the tube. The DNA was precipitated by adding 2 volumes of ethanol and placing at -70°C for 40mins. The precipitate was microfuged for 10mins, dried and resuspended in 5-10μl TE (pH 7.5).

1μl of the DNA sample was spotted onto EtBr agarose plates with standards to estimate the concentration of DNA.

3.4 Introduction of Plasmid and Bacteriophage DNA into *E.coli*

3.4.1 Transformation by $CaCl_2$

A single colony of *E.coli* cells was used to inoculate 5mls of LB medium and incubated overnight at 37°C with constant shaking. 0.5mls of this culture were used to inoculate 30mls of LB medium in a 250mls flask. The culture was incubated at 37°C with constant shaking until it reached an OD_{600} of 0.3.

The culture was transferred to a 50ml polypropylene tube and placed on ice for 30mins. The cells were pelleted by centrifugation for 10mins at 7K and 4°C, in a Sorval ss34 rotor, resuspended in 10mls of ice cold 50mM $CaCl_2$ and placed on ice for 30mins to 1 hr. The cells were again pelleted by centrifugation for 10mins at 7K and 4°C and resuspended in 2mls 50mM $CaCl_2$. 300μl of the cells were aliquoted into chilled sterile Eppendorfs.

The DNA, aproximately 30ng, was added to the 300μl of competent cells and placed on ice for 30-40mins. The cells were then heat shocked at 42°C for 2mins. 1ml of LB medium was added and the cells were incubated without shaking for 45mins at 37°C.

The appropriate dilutions were made and plated on LB plates, where an antibiotic selection was used to select for transformed cells, the appropriate antibiotic was added to the LB plates (75μg/ml).

3.4.2 Transformation by the Hanahan Method

A single colony of *E.coli* was used to inoculate 5mls of SOB medium and incubated overnight at 37°C with constant shaking. 0.5mls of this culture were then used to inoculate 30mls of SOB medium and incubated at 37°C with constant shaking until the OD_{600} reached 0.3.

The culture was transferred to a polypropylene centrifuge tube and placed on ice for 60mins, the cells were pelleted by centrifugation for 10mins at 7K and 4°C in a Sorval ss34 rotor.

The pellet was resuspended in 10mls of TFB [38], placed on ice for 30mins and the cells were again pelleted by centrifugation for 10mins at 7K and 4°C.

The pellet was resuspended in 2mls of TFB and 70μl of fresh DMSO were added to the cells, mixed thoroughly and placed on ice for 5mins. 155μl of 1M DTT were added to the cells, mixed thoroughly and placed back on ice for 10mins. Another 70μl of DMSO were added, the cells mixed and placed on ice for 5mins.

210μl samples of competent cells were transferred to chilled Eppendorfs, and approximately 30ng of DNA were added and the cells placed on ice for 30mins. The cells were then heat shocked at 42°C for 60 secs and immediately placed on ice for 1-2mins.

1ml of SOC medium was added to each tube and then they were incubated at 37°C for 60mins. The appropriate dilutions were made and the cells were

plated out on LM plates.

3.4.3 Transformation by Electroporation

A single colony of *E.coli* cells, of the strain to be transformed, was used to inoculate 10mls of 2 x YT medium and incubated overnight at 37°C with constant shaking. The overnight culture was cooled on ice for at least 10mins, in a universal bottle, and the cells were then pelleted by centrifugation for 5mins at 10,000 x *g*.

The cells were washed in 10mls cold sterile dH_2O and re-pelleted three times before resuspending finally in 50μl 10% glycerol. The final volume of cells, approximately 150μl, was aliquoted out into sterile Eppendorfs, and frozen in liquid nitrogen and stored at -70°C.

40μl of the cells were added to appropriate concentrations of the DNA to be transformed, between 10-100ng, and mixed in a Eppendorf on ice.

The cells were then carefully added to the electroporation cuvette, on ice, ensuring an even spread along the bottom.

The cells were electroporated on 2.5 and 200W and immediately after each cuvette had been treated 1ml of SOC was added and mixed carefully using a sterile pasteur pipette before transferring to a sterile glass universal.

The cells were then incubated at 37°C for 30mins with constant shaking. Dilutions of the transformation mix were then plated out on LB plates with

the appropriate antibiotic.

3.4.4 Transformation by TSS

A single colony of *E.coli* cells, of the strain to be transformed, was used to inoculate 5mls of LB medium and incubated overnight at 37°C with constant shaking. 0.5ml of this culture was then used to inoculate 30mls of LB medium and incubated at 37°C with constant shaking until the OD_{600} reached 0.3-0.4.

The cells were pelleted by centrifugation at 1000 x *g* for 10mins at 4°C and resuspended in one tenth of their original volume of ice-cold transformation and storage buffer (TSS) [18].

A 0.1ml aliquot of cells was transferred to a cold Eppendorf, mixed with 100pg of plasmid DNA and incubated at 4°C for 30mins. 0.9ml of TSS or LB medium, containing 20mM glucose, was added and the cells were incubated at 37°C with shaking for 1hr to allow expression of the antibiotic- resistance gene.

The cells in TSS were frozen immediately in dry ice and stored at -70°C until required. Frozen cells were thawed on ice and used immediately for transformation.

3.5 *In Vitro* Packaging of Bacteriophage DNA

The Freeze Thaw Lysate (FTL) and Sonic Extract (SE), either obtained from Amersham or were prepared by A. Campbell, were thawed on top of ice for

15-30mins from storage at -70°C.

The following solutions were added to an Eppendorf, on ice, in the following order;

(1) 7μl Buffer A

(2) 1-2μl DNA (200-500ng)

(3) 1μl Buffer M1

(4) 6μl SE(Optimised for different preparations.)

(5) 10μl FTL

The FTL was added as quickly as possible after the SE and mixed immediately.

The packaging reaction was then incubated for 60mins at 25°C or room temperature, diluted with 500μl SM (phage buffer) and plated out with top-agar in the presence of 10mM Mg^{2+} ions.

3.6 Identification of Recombinant DNA clones.

3.6.1 Replica Plating of Bacterial colonies

Colonies were transferred to two duplicate master plates using sterile tooth-picks and incubated overnight at 37°C. One master plate was then pressed with a sterile velvet pad and the velvet pad then pressed to a new sterile agar plate (containing selective agent where appropriate). The fresh plates were then grown overnight at 37°C or until the colonies were 2mm in width.

3.6.2 Colony Transfer on Whatmann 541 paper

A sheet of sterile Whatmann 541 paper was placed carefully onto the surface of the plate containing the colonies of interest. The colonies were approximately 1-2mm in diameter. The plate was then incubated at 37°C for 2 hours and the paper removed carefully to avoid dislodging the colonies.

The filter was then passed through the following washes [35] colony side up;

(1) 2 x 5mins	0.5M NaOH
(2) 2 x 5mins	0.5M Tris
(3) 2 x 5mins	2 x SSC
(4) 2mins	95% EtOH

The paper was then placed on 3MM paper and allowed to dry in air, ready for hybridisation.

Hybridisation could either be carried out at 65°C in 5 x SSC; 250μg/ml salmon sperm DNA or alternatively at 42°C in 50% formamide; 5 x SSC; 250μg/ml salmon sperm DNA. The filters were then washed 4 x 5mins in 2 x SSC at room temperature, dried and autoradiographed.

3.6.3 Bacteriophage plaque transfer onto nitrocellulose/nylon filters

The bacteriophage were plated out on agar plates, with 0.8% agarose in the Top agar and placed in the fridge for 30 mins before transferring.

Nitrocelluose or nylon filters were cut to the size of the plates and orientated by cutting V's in the edge of the filter.

The filters were blotted on 3MM paper, carefully saturated with 0.5M NaOH, for 2mins plaque side up and then washed in the following solutions;

(1) 1 x 20secs	0.1M NaOH/1.5M NaCl
(2) 2 x 20secs	0.5M Tris/1.5M NaCl
(3) 1 x 20secs	2 x SSC

The filters were dried between blotting paper at 37°C and baked in an oven for 2hrs at 80°C before hybridisation or crosslinking.

Hybridisation and detection were either carried out as in Section 3.7.1 or 3.7.2

3.6.4 Transfer of DNA from Agarose Gels to Nitrocellulose / Nylon filters

The gel containing the DNA to be transferred was trimmed of excess agarose and then treated at room temperature as follows;

(1) 0.25M HCL (200ml)	2 x 15mins
(2) 0.5M NaOH;1.5M NaCl (200ml)	2 x 15mins
(3) 1M Tris-HCl(pH7.5); 1.5M NaCl	2 x 15mins
(4) 10 x SSC	20mins

All of these washes were carried out whilst wearing latex gloves, with fresh solution for each step.

Nitrocellulose filters, cut slightly bigger than the area of the gel, were floated on dH_2O to wet the nitrocellulose for 90mins and then soaked in 10 x SSC immediately prior to use.

All Southern transfers with plasmid DNA were bidirectional. A glass plate was laid flat upon the bench, on top of which was laid a 5cm layer of dry paper towels, followed by 4 sheets of dry Whatmann 3MM paper, 2 sheets of Whatmann 3MM paper, saturated in 10 x SSC, a nitrocellulose/ nylon filter, the gel, a second nitrocellulose or nylon filter, 2 sheets of Whatmann 3MM paper, saturated in 10 x SSC, 4 sheets of dry Whatmann 3MM paper, a 5cm layer of dry paper towels and finally a light weight (300g) to ensure even contact.

Great care was taken to remove air bubbles between the gel and the filters.

After transfer at room temperature for at least 90mins, each filter was marked for identification at the bottom right hand corner, before baking for 2hrs at 80°C / crosslinking in Stratagene Stratalinker. The filters were then

sealed in Saran Wrap and stored at 4°C before use.

Immediately before use the filters were washed for 15mins in 2 x SSC. Hybridisation and detection were carried out as in Section 3.7.1 and 3.7.2.

3.6.5 DNA Transfer from Agarose Gels using NH_4Ac

The gel was trimmed of excess agarose and treated at room temperature in as small a container as possible as follows;

(1) 0.25M HCl (100mls)	15mins
(2) dH_2O(100mls)	10mins
(3) 1.5M NaCl:0.5M NaOH(100mls)	30mins
(4) dH_2O(100mls)	10mins
(5) 1M NH_4Ac(100mls)	1hr

All transfers were unidirectional. The gel was placed carefully on a clean glass plate and a nitrocellulose /nylon filter, cut a few mm larger than the gel and soaked in 1M NH_4Ac for a few minutes, was placed carefully on top of the gel. Care was taken to ensure that there were no air bubbles between the gel and the filter.

3 sheets of Whatmann 3MM paper, soaked in 1M NH_4Ac briefly, was placed on top of the filter, followed by a 1cm layer of dry Whatmann 3MM paper and approximately 6cm of dry paper towels. Another glass plate was placed on top and finally a light weight was added to ensure even contact.

The gel was blotted for 2hrs(or overnight) and rinsed briefly in 2 x SSC briefly before baking in a vacuum oven at 80°C for 2hrs or crosslinking.

The gel was restained to ensure the transfer was successful.

Hybridisation and detection were either carried out as in Section 3.7.1 or 3.7.2.

3.7 DNA labelling and Detection Systems

3.7.1 Radioactive Labelling

DNA probes were labelled by nick translation using α-^{32}-P-dCTP and DNA polymerase 1 (Kornberg polymerase) as described by Rigby. Nick translations were set up as follows;

DNA	100ng
10 x NT buffer	5.0μl
2mM dATP	0.2μl
2mM dGTP	0.2μl
2mMdTTP	0.2μl
α-^{32}-P-dCTP(10μCi/μl)	2.5μl
DNAse (10^{-3}M)	0.5μl
E.coli DNA polymerase 1 (10units/μl)	1.0μl
sterile dH_2O	$\uparrow$ 50μl

Once prepared, the nick translation mixture was incubated at 16°C for 90mins. The reaction was then stopped by the addition of 5μl of 0.2M EDTA.

Unincorporated α-^{32}-P-dCTP was separated from labelled probe by the spun column procedure described by Maniatis [76].

A 1ml disposable syringe was plugged at the bottom with a small amount of sterile polyallomer wool. The syringe was then filled with Sephadex G-50 equilibrated in TE(pH 8.0), containing 0.1M NaCl(STE). The syringe was compacted by centrifugation in a bench centrifuge for 5mins at 3,000g and subsequently topped up until it was approximately 0.9ml bed volume.

0.1ml of 2 x SSC was added to the column and recentrifuged at exactly the same speed as above, to equilibrate the column. The wash in 2 x SSC was repeated once more before adding the nick translation mixture to the column which was made up to 0.1ml with dH_2O.

The column was recentrifuged as before and the 0.1ml effluent from the column was collected in a decapped sterile Eppendorf tube. The unincorporated α-^{32}-P-dCTP remains in the syringe.

1μl of the nick-translation was removed before and after the spun column procedure and counted by Cerenkov counting in a liquid scintillation counter. A comparison of the readings from before and after the spun column procedure allowed an estimate of the percentage incorporation of label and the specific activity of the probe.

The probe was denatured by boiling in a water bath for 10mins and placing immediately on ice, before adding to the prehybridisation buffer.

The filters to be probed were prehybridised and hybridised in the following solution, in sealed plastic bags;

Dextran sulphate	2g
10 x SSC	10mls
50 x Denhardt's solution	2mls
10% SDS	0.2ml
0.45M EDTA(pH 8.0)	0.1ml
Salmon sperm DNA	0.4ml
Sterile dH_2O	↑ 20mls

The solution was heated to 65°C before adding to the filters and prehybridisation was carried out at 65°C for 4hrs.

The radioactive probe was denatured by heating to 100°C for 10mins before adding to the prehybrisation mix in the plastic bag. The probe was thoroughly mixed and air bubbles expelled before resealing the bag. Hybridisation was allowed to proceed overnight at 65°C.

After hybridisation, the filters were washed successively with the following solutions at 65°C;

(1) 5 x SSC; 0.1% SDS	30mins

(2) 2 x SSC; 0.1% SDS	60mins
(3) 1 x SSC; 0.1% SDS	30mins

The filters were then blotted dry on Whatmann 3MM paper and sealed in Saran wrap. Each filter was autoradiographed by exposure to Fuji-RX X-ray film in Okamoto X-press cassettes using Mach II intensifier screens at -70°C for between 12hrs-8 days. Autoradiographs were developed using a X-omat automatic processor.

To reprobe, the filters were first washed in preboiled 0.1% SDS and autoradiographed to ensure no probe remained on the filter.

3.7.2 Non-Radioactive Labelling

The probe was prepared from approximately 200ng of DNA, either isolated fragment or plasmid, the typical reaction mixture is described below.

The DNA was added to the dH_2O first, boiled for 10mins in a waterbath and placed immediately on ice to denature the DNA. Once cooled the DNA was microfuged and the other components of the reaction added.

DNA	200ng
Hexanucleotides	2μl
dNTP labelling mix	2μl
Sterile dH_2O	$\uparrow$ 20μl
Klenow	0.5μl (5u/μl stock)

The reaction was incubated in a 37°C water bath overnight before adding the following;

0.2M EDTA	2μl
3M NaAc	1.8μl
EtOH	72μl

The reaction was mixed well and placed either at -70°C for 30mins or -20°C overnight to precipitate the DNA.

The DNA was pelleted by microfuging for 10mins, washed in 70% EtOH, dried and resuspended in 50μl TE and 1μl 5% SDS by placing at 37°C for 10mins and vortexing briefly on 0, 5 and 10mins. The probe was stored at -20°C.

Probe yield was estimated using serial dilutions of the probe in Buffer 3, ranging from 10^{-1} to 5 x 10^{-5}. 1μl of each of the probe dilutions was spotted onto a piece of nylon filter on a 2 x SSC pad, along with the BCL control dilutions after boiling for 10mins to denature the DNA. The DNA was UV crosslinked or baked onto the filter before the detection step.

The detection of labelled DNA on the filter involved the following washes at room temperature, in the smallest container possible;

(1) Washing buffer(100mls)	1-5mins
(2) 1% Block (100mls)	30mins

(3) AB Conjugate(30mls)	30mins
(4) Washing buffer(2x 100mls)	2 x 15mins
(5) Buffer 3(100mls)	1-5mins
(6) AMPPD(20mls)	5mins

Excess liquid was allowed to drip off the membrane and the filter was blotted dry. The filter was sealed in Saran Wrap and placed at 37°C for 5-15mins before exposing to film.

Each filter was autoradiographed by exposure to Fuji-RX X-ray film in Okamoto X-press cassettes using Mach II intensifier screens at room temperature for between 30mins-4hrs. Autoradiographs were then developed using a X-omat automatic processor. The filters were active for 24hrs after detection.

The probe concentration was calculated by matching the intensity of the probe dilutions with the BCL control dilutions when 1ng of probe DNA is equivalent to 260ng of BCL in 1μl.

The optimum concentration of probe for hybridisation is between 10-15ng/ml of hybridisation buffer.

The filters to be probed, see Sections 3.6.3, 3.6.4 3.6.5 were prehybridised and hybridised in the following solution;

SSC	5 x

Formamide	50%
N-lauroyl sarcosine	0.1%
SDS	0.02%
Block	2%
H_2O	↑ total volume

Prehybridisation was carried out at 37°C with constant shaking for at least 1hr and required 20mls/100cm^2 of filter. For plaques or colonies prehybridisation required 6mls/filter, if more than 2 filters, one filter being equivalent to 52.8cm^2.

Hybridisation was carried out at 37°C with constant shaking overnight and required 2.5mls/100cm^2 of filter and 10-20ng/ml DIG labelled probe, denatured by boiling for 10mins and placing immediately on ice.

After hybridisation the filters were washed in the following solutions, at room temperature;

(1) 2 x SSC ; 0.1% SDS	2 x 5mins
(2) 1 x SSC ; 0.1% SDS	2 x 15mins

The filters were then put through the washes described previously when estimating probe concentration, to detect the labelled DNA on the filter.

To reprobe, the filter was washed briefly in dH_2O, soaked for 2 x 15mins in 0.2M NaOH; 0.1% SDS at 37°C and washed briefly in 2 x SSC. The filter

could be stored dry sealed in Saran wrap or with TE sealed in a plastic bag or could be transferred immediately to prehybridisation buffer.

3.8 Analysis of Recombinant DNA clones

3.8.1 Restriction Digests

Restriction digests typically contained 0.2-1.0μg of DNA in a volume of 20μl or less, this was varied i.e. larger amounts of DNA were used in a larger total volume.

In general, the following was added to a sterile Eppendorf: approximately 1μg of the DNA to be digested; 2μl of the appropriate 10x digestion buffer, specific to the requirements of the enzyme; sterile distilled H_2O to make the volume up to 20μl. Finally, 1 unit or 1μl of restriction enzyme was added and the Eppendorf microfuged to ensure the contents were at the bottom of the tube.

The tube was then incubated for 90mins in a 37°C waterbath and the reaction stopped by adding 0.5M EDTA (pH 7.5) to a final concentration of 10mM.

When necessary a small sample of the digest (approximately 0.1μg) was removed before adding the EDTA and run on a minigel to check the reaction had gone to completion.

3.8.2 Double Digests

For double digests a compatible buffer, giving 100% activity for both enzymes, was used if possible and when there was no compatible buffer the DNA was digested as described above, a sample was removed to check this digest had gone to completion and the DNA was either phenol extracted and ethanol precipitated or precipitated with isopropanol as follows:

3.8.3 Isopropanol Precipitation

An equal volume of 4M Ammonium Acetate was added to the restriction digest, and the tube microfuged to collect everything at the bottom of the tube. 1x the total volume of isopropanol was added and the tube placed at room temperature for 10mins to precipitate the DNA.

The DNA was pelleted by microfuging for 10mins and the supernatant removed by aspiration. 100μl of 70% EtOH was added to wash the pellet, vortexed briefly and again microfuged for 10mins to pellet the DNA. The EtOH was removed by aspiration, the pellet dried and resuspended in new restriction buffer for the next digest.

3.9 DNA Ligations

Both vector and insert DNA were purified and digested with restriction enzymes as appropriate and the restriction enzymes were removed by either phe-

nol extraction and ethanol precipitation or isopropanol precipitation. The DNA was resuspended in ligation buffer [64] or TE. For plasmid and bacteriophage ligations the optimum molar ratio for ligations was found to be 3:1 insert:vector. The appropriate molar ratios of vector to insert were calculated using the following equation;

$$\text{Insert (g)} = \text{Vector(g)} \times \frac{Length\,of\,Insert\,(kb)}{Length\,of\,Vector\,(kb)} \times \text{Molar ratio (3/1)}$$

The standard ligation reaction was as follows:

Ligation buffer(x10)	2μl
Insert:Vector DNA	3:1
Sterile dH_2O	$\uparrow 20\mu$l
T4 DNA Ligase	2μl or 2 units

The ligation mix was placed at room temperature for between 4hrs to overnight. The ligation was tested by running a small sample on a gel alongside suitable controls.

3.10 Blunt Ending

Fragments with protruding 5′ ends are converted to blunt ends using the DNA polymerising activity of the Klenow fragment of *E.coli* DNA polymerase I.

The reaction mixture was set up as follows:

Restriction Fragment	↑ 1μg in 10μl
2mM dCTP	1μl
2mM dATP	1μl
2mM dGTP	1μl
2mM dTTP	1μl
Nick Translation Buffer(10x)	2.5μl
Sterile dH_2O	↑ 25μl

2 units of the klenow fragment of DNA polymerase I were added, the tube was mixed and incubated for 15-30mins at 22°C. The enzyme was then inactivated by heating at 70°C for 5mins. The DNA is then ready for any ligation reactions since end-filling and ligation reactions can be carried out sequentially in the same reaction mixture.

3.11 Dephosphorylation of DNA

The DNA was digested to completion with the required restriction enzyme, extracted once with phenol:chloroform and precipitated with EtOH. The dried DNA pellet was resuspended in a minimum volume of 10mM Tris.Cl (pH 8.0) and added to the following reaction mixture in a sterile Eppendorf;

CIP Buffer(10x)	5μl
Sterile dH_2O	↑48μl

The amount of Calf Intestinal alkaline Phosphatase (CIP) required for the reaction was calculated on the basis that 0.01 units of CIP will remove the terminal phosphates from 1pmole of 5′ ends of DNA where 1pmole of 5′ ends of a 4kb linear DNA molecule is 1.6μg of DNA.

After adding the appropriate amount of enzyme to dephosphorylate the protruding 5′ ends, the reaction mixture was incubated at 37°C for 30mins. A second aliquot of enzyme was added and the incubation continued for a further 30mins. 40μl of dH_2O; 10μl of 10 x STE and 5μl of 10% SDS were added to the reaction mixture and it was placed at 68°C for 15mins to denature the enzyme.

The enzyme was removed with two phenol:chloroform extractions followed by two chloroform extractions and the DNA precipitated with EtOH, dried and resuspended in the desired buffer.

N.B.

(1) CIP is usually supplied as a suspension of Ammonium Sulphate, this has to be removed if the DNA is required for kinasing or it will be precipitated by the EtOH and the ammonium ions will interfere with the polynucleotide kinase. This can be achieved by passing the reaction mixture through a spun column of Sephadex G-50 equilibrated with TE.

(2) If heat inactivation of CIP in the presence of SDS is insufficient, trinitroloacetic acid can be added to a final concentration of 10mM to chelate the

divalent zinc ions resulting in the enzyme being more sensitive to heat.

3.12 Maintenance of Stocks

3.12.1 Preparation of Glycerol Stocks

A single colony of *E.coli* cells was used to inoculate5mls of LB medium and incubated overnight at 37°C with constant shaking. 1.5mls of the culture were transferred to a sterile Eppendorf and microfuged for 1min. The supernatant was removed by aspiration and the cells resuspended in 0.5ml of fresh LB medium.

3.12.2 Preparation of Phage Lysates of Bacteriophage lambda

A single plaque of the desired phageλ was picked into 1ml of phage buffer and a drop of $CHCl_3$ was added. Serial dilutions of this stock were made (10^{-1} - 10^{-3}) and and 100μl of each were mixed with 200μl plating cells, made from a λ sensitive strain. The phage were allowed to absorb at 37°C for 20mins and were then plated out in LB top agar on LB plates. The plates were incubated at 37°C for 8-16hrs. Growth was halted when the plaques were confluent at the optimum dilution of phage. 4mls of phage buffer were added to the plate and placed at 4°C for several hours, with some swirling of the solution. The buffer was then collected, a drop of $CHCl_3$ added and the lysate vortexed. The lysate was centrifuged at 4K for 5mins to remove cell debris and the supernatant stored at 4°C.

Plating cells

A single colony of a phage λ sensitive *E.coli* strain was inoculated into 30mls of LB medium containing 0.2% maltose. The culture was grown overnight at 37°C with constant shaking. The cells were harvested by centrifugation at 7K for 5mins and resuspended in 1/10th of the original volume of 0.01M $MgSO_4$. The plating cells were then stored at 4°C for up to 14 days.

3.12.3 Preparation of Phage Lysates of Bacteriophage P1

A single colony of *E.coli*, of the strain required to transduce, was inoculated into 5ml LB medium and grown overnight at 37°C with constant shaking.

A drop of the fresh overnight culture was used to inoculate5mls LB medium containing 5 x 10^{-3}M $CaCl_2$ and grown to an OD_{600} of 1(2 x 10^8 cells/ml).

The P1 phage was preabsorbed by adding 10^7 phage to 1ml of the culture and incubating for 20mins in a 37°C waterbath. The phage was then plated out with 2.5mls of R top-agar on R plates and incubated face up for 8hrs at 37°C.

The soft top agar layer was scraped off the plate into a centrifuge tube and the surface of the plate washed with 1ml of LB medium into the centrifuge tube.

5 drops of chloroform were added to the contents of the tube, vortexed for 30secs and placed at room temperature for 10mins.

The cell debris was pelleted by centrifugation, 4K 15mins, and the supernatant (lysate) transferred to a small universal bottle. The lysate was then titred with serial dilutions of 10^{-7}, 10^{-8} and 10^{-9}. 0.1ml of each dilution was mixed with 200μl of an overnight of the strain from which the lysate was made, incubated for 8mins in a 37°C waterbath and plated out on R plates with R top-agar.

3.13 P1 Transduction

A single colony of *E.coli*, of the strain to be transduced, was inoculated into 5mls LB medium and grown overnight at 37°C with constant shaking.

The 5ml overnight was centrifuged, 4K 15mins, the supernatant removed by aspiration and the pellet resuspended in 5mls MC buffer. The culture was then incubated for 15mins in a 37°C waterbath.

0.1ml of this culture was then added to 5 test tubes containing the following;

(1) 0.1ml P1 lysate 10°

(2) 0.1ml P1 lysate 10^{-1}

(3) 0.1ml P1 lysate 10^{-2}

(4) 0.1ml P1 lysate 10^{-3}

(5) No P1 lysate (control)

As another control a tube was prepared with 0.1ml of P1 lysate 10° with no cells.

The tubes were incubated for 20mins in a 37°C waterbath and 0.2ml of 1M sodium citrate was added to prevent multiple absorption.

The contents of the tube were mixed with F top-agar, plated on minimal plates and incubated at 37°C for 48hrs.

3.14 Sequencing

All sequencing was carried out using the Sequenase Kit from USB.

The use of culture grade DMSO at 10% in the sequencing reactions was recommended to enhance the intensity of the signal and to reduce background, presumably by preventing secondary structure from forming.

3.14.1 Double Stranded Sequencing

To anneal the primer and template, the following reaction was set up;

DMSO	1μl
Reaction Buffer(USB)	2μl
DNA template	5μl(50ng/μl)
Primer	1μl(1μg)
Sterile dH_2O	$\uparrow$ 10μl

The reaction was boiled for 3mins to denature the DNA template and immediately snap-cooled on ice to minimise renaturation.

For sequencing close to the primer (10-200bases), the normal labelling mix (USB) diluted 1/20 in distilled water, was used and for sequencing up to 400bases from the primer a dilution of 1/5 was used.

The following was added to the cooled annealing reaction to label the DNA;

0.1M DTT (USB)	1μl
α-^{35}S-dATP(1000Ci/mM, Amersham)	1μl
labelling mix (1/20 or 1/5)	1μl
Sequenase(1/3 in enzyme dilution buffer(USB))	1μl

The labelling reaction was incubated at room temperature for 2-5mins.

To terminate the labelling reaction, 3.5μl of the above reaction was added immediately to 4 tubes containing 2μl of the appropriate termination mix i.e. 2μl of ddGTP, ddATP, ddTTP, ddCTP.

The tubes were incubated at 37°C for 5mins before adding 4μl of Stop solution (USB) to each tube. The reactions were stored at 4°C and used within 1 week.

The samples were placed at 80°C for 2mins just before loading onto the pre-warmed gel.

3.14.2 Preparation of Sequencing Gels

Sequencing gels were prepared between 2-20hrs before use.

The gel plates were thoroughly cleaned, ensuring any remaining siliconiser was removed using acetone / xylene. The plates were then washed 2 x with EtOH, and once with acetone.

Siliconiser should then be applied evenly over one of the plates (always the same one).

8% gels were generally used, 6% gels were used to give better resolution further away from the primer. A 50ml 8% gel was prepared as follows:

ACS Urea	25g
10 x TBE	5ml
30% Acrylamide	10ml
10% NH_4 persulphate	0.4ml
dH_2O	15ml

The 30% acrylamide was prepared weekly and the 10% NH_4 persulphate was prepared the same day.

The urea was dissolved by funnelling hot water over the flask, the gel mix was then degassed and 15μl of TEMED was added to catalyse the polymerisation.

The gel was poured onto the plates and the sharks tooth comb inserted

upside down. The sides of the gel were clamped with bulldog clips and the gel left at room temperature to set.

Once the gel was set, the comb was removed and any urea crystals present were washed out using 1 x TBE. The comb was replaced with the teeth downwards to form wells and the gel was pre-run at 30-40W for 15-30mins before loading the samples.

The samples were placed at 80°C for 2mins before loading onto the gel and the gel run at betwwen 30-40W for 2.5-3hrs.

Following electrophoresis the top plate was removed and the gel on the lower plate was fixed by soaking in 7% acetic acid; 7% methanol (1l) for 2 x 15mins, diluting the urea to less than 1%, which decreases the stickiness of the gel and abolishes the quenching effect of urea on ^{35}S.

After fixing the gel is drained and a piece of Whatmann 3MM paper cut to size was placed over it and smoothed down on the gel carefully. The back plate is then removed leaving the gel stuck to the paper.

The gel is dried on a vacuum drier for 1-1.5hrs, and exposed to Kodak XAR-5 or Agfa Curix film for 24-48hrs.

3.15 Trimethoprim Selection

A single colony of the *E.coli* strain required to be mutated to *thy*A$^-$ is inoculated into 5mls of 1 x A medium containing glucose; Mg_2SO_4 and any necessary supplements and grown overnight with shaking at 37°C. 200μl of

this saturated culture was used to inoculate 5mls of the same medium with 200μg/ml thymidine and 200μg/ml trimethoprim and grown overnight with shaking at 37°C.

50μl of this culture was used to inoculate 5mls of identical medium and again grown overnight with shaking at 37°C. The overnight culture was diluted and the dilutions were plated out on LB plates supplemented with 200μg/ml thymidine to obtain single colonies. The colonies were replica plated from a master plate onto glucose minimal plates with and without thymidine and incubated at 37°C overnight and screened for *thy*A$^-$ colonies. These colonies were picked and purified and the rest of the strain's background checked.

An alternative method used was to spread a drop of overnight culture onto glucose minimal plates supplemented with 50μg/ml thymidine and 10μg/ml trimethoprim and incubate for 36hrs at 37°C. Small colonies were picked and purified onto the same medium and the resulting colonies tested for the *thy*$^-$ phenotype.

3.16 Penicillin Enrichment

The mixture of *trp*$^-$/$^+$ cells were washed and then resuspended in 5mls of minimal medium supplemented with 100μg/ml of tryptophan and any other known requirements and grown overnight with shaking at 37°C.

The culture was washed twice with minimal medium and resuspended in 5mls minimal medium. The density of cells in the medium was estimated

using a spectrophotometer at OD_{600} and 5mls of pre-warmed glucose minimal medium with supplements, other than tryptophan, was inoculated with enough bacteria to give a final concentration of 1-2 x 10^7cells/ml and the culture incubated at 37°C with shaking. An OD_{600} reading was taken immediately after inoculation and the culture monitored until the optical density increased 4-5 fold to allow the culture to be starved for tryptophan.

Ampicillin was then added to a final concentration of 20μg/ml and the culture was incubated for a further 60-90 mins or until lysis is complete. This was observed as a drop in the OD_{600} reading.

After lysis the culture was washed in minimal medium and resuspended in 5mls of glucose minimal medium supplemented with tryptophan and incubated at 37°C overnight with constant shaking.

The procedure was repeated exactly as before using the penicillin treated overnight culture. The resulting culture was diluted and the dilutions were plated on glucose minimal plates supplemented with tryptophan. Single colonies were picked and purified and their *trp* phenotype tested by replica plating onto glucose minimal with and without tryptophan. Possible *trp*$^-$ colonies were re-purified and retested on the same plates as well as testing the background of the strain.

3.17 Preparation of Lysogens

A high titre lysate (10^{10} to 10^{11} pfu/ml) is required and was prepared as described in the methods Section 3.12.2 for the specific phage to be made lysogenic.

100μl of plating cells in Mg_2SO_4, prepared as described in methods Section 3.12.2 were mixed with approximately 10^9 phage and incubated for 20mins at 37°C. A cells only control was also incubated. A 100 fold dilution was made of both cultures in LB and the diluted culture was incubated at 32°C for 45mins. The culture was centrifuged for 5g for 10mins and the cell pellet resuspended in 200μl LB. 100μl of each culture were transferred to a test-tube containing 100μl of phage lysates of λvir (λNM63 and λNM220) and incubated at 37°C for 20mins. 3mls of LB top-agar were added and the contents of the tube plated out on LB plates. The plates were incubated at 32°C overnight.

100μl of the cells only control was also plated out directly without mixing with the phage lysate of λvir.

The resulting colonies were picked and purified on LB at 32°C and tested for lysogeny by streaking the colonies on duplicate plates and incubating them at 32°C and 42°C. If the colony was sensitive to the temperature increase in that it showed lysis, then the colony was designated lysogenic.

3.18 Preparation of Cured Cells

A single lysogenic colony of *E.coli* was streaked out on LB plates and incubated at 32°C overnight. A single colony was then streaked out on LB plates and incubated at 37°C overnight. Most of the colonies lyse but the few colonies which were cured were picked, purified and retested by plating at 42°C.

3.19 Preparation of Phage λ crosses

3.19.1 With plasmid

A high titre lysate (10^{10} to 10^{11} pfu/ml) is required and was prepared as described in the methods Section 3.12.2 for the specific phage to be crossed. Plating cells in Mg_2SO_4 were prepared as described in the methods Section 3.12.2 from the strains of *E.coli* carrying the plasmid of interest. A 100μl of the plating cells were mixed with phage at a m.o.i. of 1 [67]. The phage were absorbed by incubating at 37°C for 20mins,plated out in LB top agar onto LB plates and incubated overnight at 37°C.

3.19.2 With another phage

A high titre lysate (10^{10} to 10^{11} pfu/ml) is required and was prepared as described in the methods Section 3.12.2 for the specific phages to be crossed.

A 100μl of concentrated (2:1) plating cells in Mg_2SO_4 prepared as described in the methods Section 3.12.2 were mixed with phage to a m.o.i. of 5 for both

phage types. The phage were adsorbed by incubating at 37°C for 20mins, the culture was diluted 1/100 in warm LB broth and incubated at 37°C for 30-60mins to allow recombination to take place. A few drops of $CHCl_3$ were added to lyse the cells.

100μl of the phage lysate was added to a suitable host strain of *E.coli*, allowing selection for recombinant phage, incubated at 37°C for 20mins to absorb phage. 3mls of LB-top agar were added and the cells plated out on LB plates. The plates were then incubated at 37°C overnight at appropriate temperature for selection to occur.

3.20 Identification of ampicillin resistant plaques

3.20.1 Nitrocefin; plate method

A working solution of nitrocefin was prepared as described in the Appendix [85]. The solution was dropped onto λ plaques on solid media. Colour production was immediate but was left at room temperature for at least 5mins. β-lactamase-producing plaques showed a change in colour from yellow to red. Nitrocefin has only a low level of antibacterial activity so that treated colonies/plaques could be subcultured as usual.

A λ AMP^r (NM1134) was used as a positive control and a λ AMP^s was used as a negative control.

3.20.2 Ampicillin plates

Dilutions of phage lysates were spotted onto a lawn of an ampicillin sensitive *E.coli* strain plated in LB-top agar on a LB plate supplemented with ampicillin and incubated at 37°C overnight. β-lactamase-producing plaques were identified by producing a halo of cells. These plaques were picked, purified and retested.

A λ AMP^r (NM1134) was used as a positive control and a λ AMP^s was used as a negative control.

3.21 Identification of *trp*$^+$ phage

Dilutions of phage from packaged ligation mixes were spotted onto lawns of different *E.coli trp* mutants; NM311 *trpA*$^+$; NM312 *trpC*$^+$ NM127 *trpB*$^+$, plated in F-top agar on glucose minimal-ACH plates. *trp*$^+$ plaques were identified by producing a halo of cells around them. These plaques were picked, purified and retested.

(N.B. It was important to use only agar obtained from Difco in these experiments.)

3.22 Batch Culture

A single colony of the strain of interest was inoculated into 30mls of minimal medium with a single carbon source of choice and necessary supplements. The culture was grown overnight at 37°C with constant shaking and then cen-

trifuged at 4K for 10mins. The cell pellet was washed in pre-warmed minimal medium, centrifuged again and resuspended in pre-warmed minimal medium. The concentration of cells in the culture was estimated by measuring the OD_{600} and approximately 10^7 cells/ml were inoculated into fresh pre-warmed culture medium with the carbon source of choice and necessary supplements. The growth of the culture was monitored by sampling some of the culture, every hour for 6-8hrs and taking OD_{600} readings from these aliquots. The growth curves were plotted with the OD_{600} readings against time.

3.23 β-Galactosidase Assay

3.23.1 Hydrolysis of ONPG

O-nitrophenyl-β-D-galactopyranoside (ONPG) is hydrolysed in the presence of β-galactosidase, from a colourless compound to o-nitrophenol, a yellow compound, which can be measured by its absorption at OD_{420}.

An aliquot (0.1-0.5ml) of fresh cell culture to be assayed was added to Z-buffer (to 1ml) in a small test-tube, 2 drops of $CHCl_3$ and 1 drop of 10% SDS were added and the cells vortexed. The tube was then incubated at 28°C for a few mins to equilibrate before adding 0.2ml of an ONPG solution (4mg/ml). The time taken for a yellow colour to develop was noted and the amount of ONPG produced i.e. the absorbance at OD_{420} was measured. The cell density of the culture was estimated by reading the absorbance at OD_{600} and the

contribution to the absorbance due to light scattering was corrected for, using the absorbance at OD_{550}.

The units of β-galactosidase activity present were calculated as described in the equation;

$$\text{Units} = 1000 \text{ x } (OD_{420} - (1.75 \text{ x } OD_{550}))/(t \text{ x } v \text{ x } OD_{600})$$

Where OD_{420} and OD_{550} were read from the assay tube, OD_{600} was measured from the cell culture before the assay and t is the time of the reaction and v is the volume of cells added to the assay tube.

The units of β-galactosidase are calculated as the increase in ONP per minute per bacterium.

3.24 Permease Assay

3.24.1 The O-nitrophenyl-β-D-thio-galactopyranoside (TONPG) Selective Medium

TONPG selects against *lac* permease function. It is lethal to the cell in high internal concentrations [37]. It is not an inducer of the operon or a good substrate for β-galactosidase, but is transported into the cell via the *lac* permease. Its selective effect is greatest on succinate or acetate as the sole carbon source.

The medium used in the selection experiments is described in the appendix;

TONPG was used at a final concentration of 1-2 x 10^{-3}M in Hobson's medium with sodium succinate as the carbon source. The plates were incubated for 72hrs at 28°C. IPTG (10^{-3}) or IAA (varying concentrations) were added as inducer of the permease under *lac* promoter control or *trp* promoter control respectively.

Colonies to be tested were replica plated onto plates containing TONPG and plates without TONPG. *Lac* Y^+ colonies were selected as those which could grow in the absence of TONPG but were killed when TONPG was present.

3.24.2 Rate of Accumulation of ONPG

A single colony of the strain to be assayed for permease activity was inoculated into 30mls of minimal succinate medium with the neccessary supplements and grown overnight at 37°C with constant shaking. The cultures were centrifuged at 4K for 10mins in a Sorval ss34 rotor and washed once with minimal succinate medium and resuspended in 5mls minimal succinate medium. The concentration of cells in this culture was estimated by measuring the OD_{600} and approximately 10^7 cells were used to inoculate fresh culture medium (10mls). A series of cultures were set up containing different concentrations of the inducer, IAA. β-galactosidase activity was induced in all experiments using a constant concentration of IPTG (10^{-3}M). The cultures were grown at 37°C, with constant shaking, for several hours to ensure induction of both proteins.

An aliquot of the culture was placed on ice to stabilise the permease. The concentration of cells was estimated by measuring the OD_{600} and the cells diluted if necessary to an OD_{600} of approximately 0.15 for more accurate measurements of uptake. 0.9mls of culture were added to a cuvette and 0.1ml of ONPG (4mg/ml) were added and equilibrated to 37°C. The rate of conversion of ONPG to ONP was monitored at OD_{420} (37°C). The initial rate of increase in absorbance was determined to be proportional to the rate of uptake of the galactoside [65, 25].

The units of permease were calculated as follows;

$$\text{Units} = 1000 \text{ x } (\Delta OD_{420})/(t \text{ x } v \text{ x } OD_{600})$$

Where ΔOD_{420} is the increase in absorbance in the reaction mixture, t is the time to achieve the change in OD_{420} and v is the volume of culture added to the reaction mixture.

The units of permease activity were calculated as units per minute per bacterium.

Control strains were also assayed to ensure the increase in absorbance was not due to diffusion (614*lac*Δ *Y*) or IPTG induction (614) or a change in the strain background due to the genetic manipulations (614(5)trp^+).

4 Subcloning The Lactose Permease

4.1 Introduction

As discussed in Chapter 1, *lacZ* and *lacY* gene expression, from different controlling sequences, was required to allow independent and variable expression. To achieve this it was necessary to find a promoter other than the *lac* promoter which could induce genes over a similar range i.e. 1,000-10,000 fold.

A variety of vectors have been described which allow the regulated expression of cloned genes in *E.coli* [94]. Of these vectors, most contain promoters which give high level constitutive expression. For induction, the most commonly used is the *lac* promoter [33], or a combination of the *lac* and *trp* promoters, the *tac* promoter [26, 96]. Both of these inducible promoters are unsuitable for the purposes of this work because expression would not be independent of *lac* promoter induction.

A suitable candidate for the second promoter was the *trp* operon promoter alone [106, 96, 84]. The *trp* promoter, like that of *lac*, is responsible for coordinate expression of more than one gene i.e. the *trp* biosynthetic operon [116, 78]. This promoter is also inducible, but differs from the *lac* system in that the operon is switched on and the genes expressed in the absence of tryptophan (or when the level of tryptophan is very low).

4.2 The Tryptophan Operon

The entire operon has been cloned and sequenced [117]. It comprises of 5 structural genes which share the same controlling sequences [117, 116]. The cluster of genes is transcribed into a single mRNA from which the individual proteins are translated. The structural genes; E,D,C,B and A, encode for Anthranilate synthetase Component I; Anthranilate synthetase Component II ;PR Anthranilate isomerase; Tryptophan synthetase β-sub-unit and Tryptophan synthetase α-sub-unit respectively. These proteins are involved in the conversion of chorismate into tryptophan, [116]; The DNA following the last structural gene of the *trp* operon contains a complex set of termination signals. *In vivo*, the mRNA transcribed from the *trp* operon ends efficiently at a termination site *trpT*, 36bp past *trpA*.

4.2.1 The *trp* Aporepressor

Control of the operon is mediated through the *trp* aporepressor protein, encoded by the *trpR* gene which is situated approximately 30 mins from the *trp* operon on the *E.coli* chromosome [97, 103]. The *trp*R gene has its own promoter and terminator sequences and therefore forms an independent transcription unit. The gene has been cloned and sequenced, [117] and the protein has been purified [89].

The *trp* aporepressor has been demonstrated to have only weak, nonspe-

cific affinity for DNA [15] but on binding the co-repressor, L-tryptophan, the repressor binds to the *trp* operator DNA with high affinity [15, 116]. *In vitro* binding studies have demonstrated that binding of the *trp* repressor and RNA polymerase are mutually exclusive [89, 15].

4.2.2 The *trp* Attenuator

A transcribed leader region, 162bp downstream of the transcription initiation site, *trpL*, contains a regulated site of transcription termination, the attenuator [116, 78]. The leader region of the *trp* operon encodes a 14-residue peptide with adjacent tryptophan residues at positions 11 and 12. The transcript of the leader region can form alternate secondary structures which are believed to determine whether transcription terminates or continues through the attenuator.

4.3 Regulation of *trp* Operon Expression

trp operon expression is influenced by both specific and general mechanisms. The level of repression and attenuation of transcription reflects the tryptophan pool size, which in turn is determined by biosynthetic and catabolic fluxes. General effects of growth rate on RNA and protein synthesis and possibly on the rate of RNA degradation also affect expression [80, 81, 82, 6]. For the purpose of this thesis only transcriptional regulation is of interest although other factors will be taken into account where relevant.

Figure 9: Factors Affecting *trp* Operon Expression I

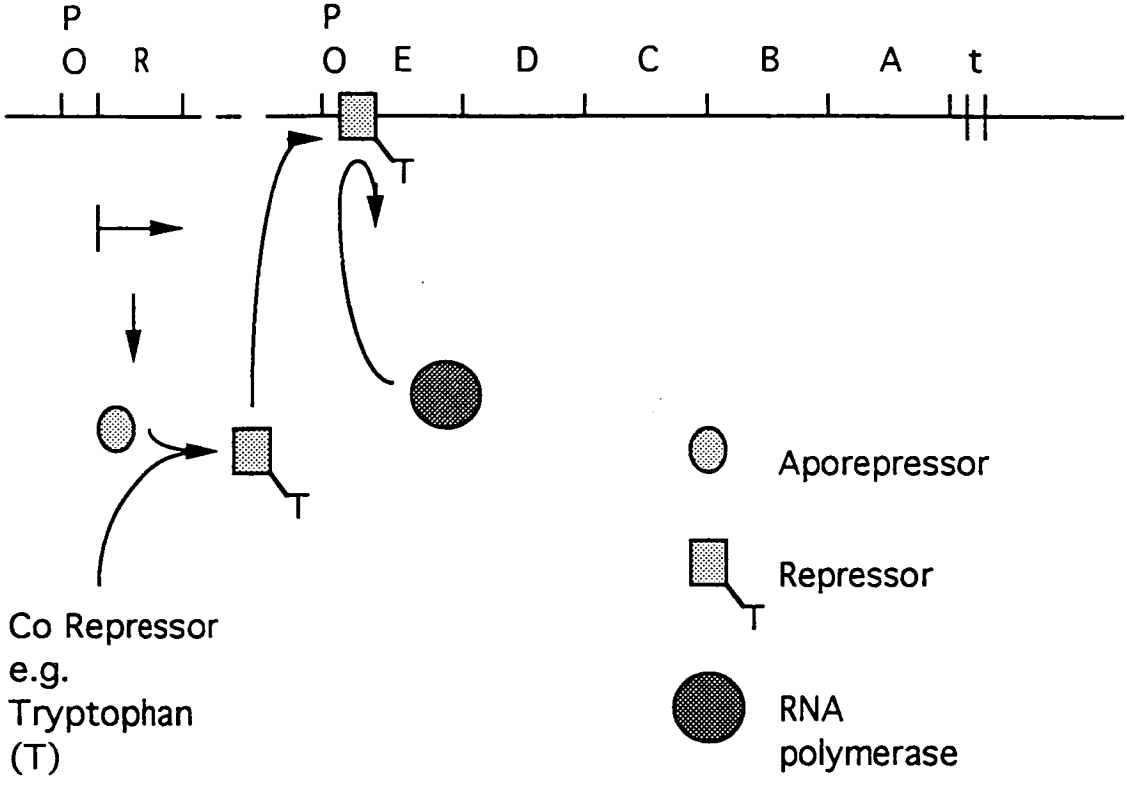
(1)**Tryptophan Present**; Tryptophan binds to the aporepressor and induces a conformational change to form the repressor. The repressor binds to the promoter/ operator region and prevents transcription.

(2)**No Tryptophan Present**; In the absence of a co-repressor such as tryptophan, the aporepressor is unable to bind to the promoter/ operator and RNA polymerase can bind and transcription occurs.

(3)**Intermediate Tryptophan Levels**; An equilibrium between the aporepressor and tryptophan and functional repressor occurs. The level of transcription is dependent on the level of functional repressor and its interaction with the promoter/ operator.

Figure 9 :: Factors affecting *trp* operon expression

(1) Tryptophan Present:: No Transcription



(2) No Tryptophan Present:: Transcription

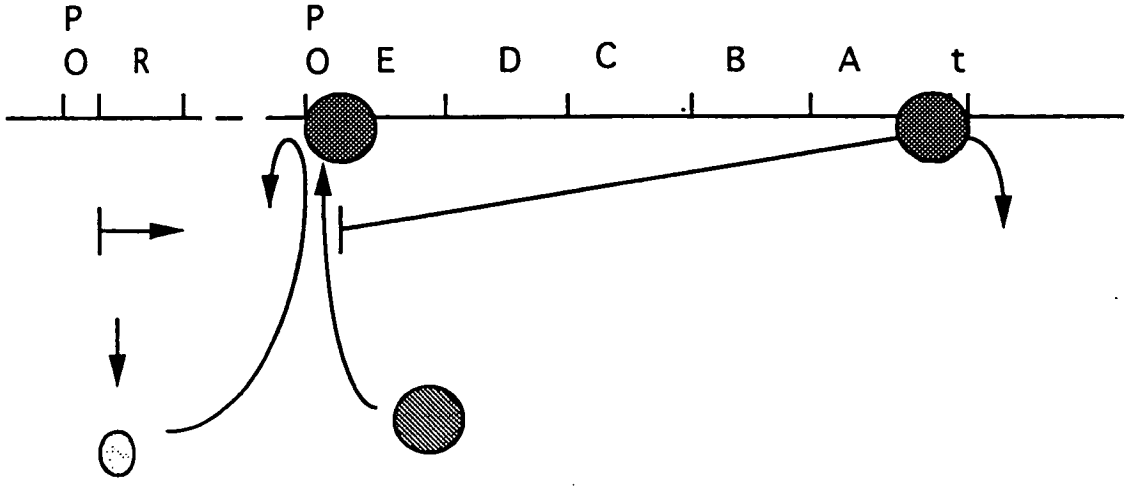


Figure 9:: (continued)

(3) Tryptophan present at low/intermediate levels:: Equilibrium

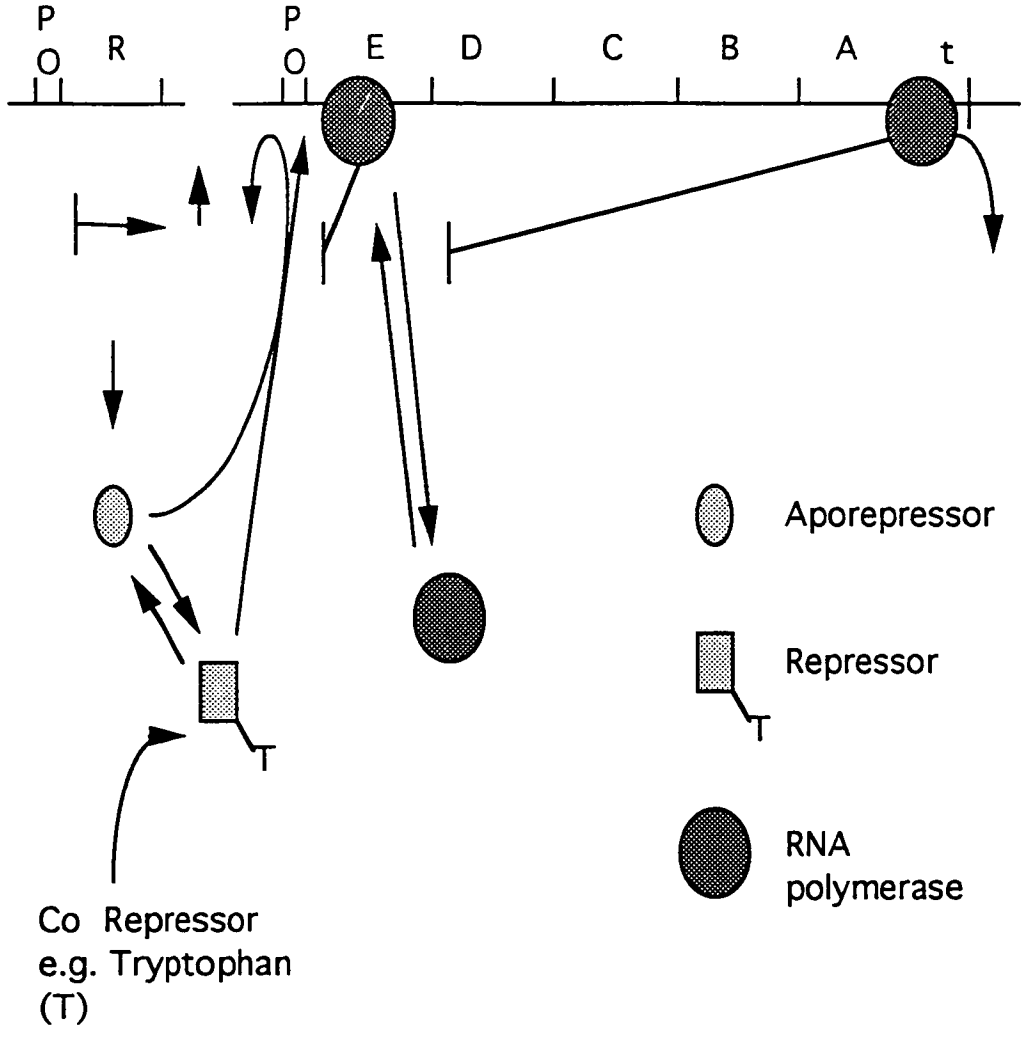


Figure 10: Factors Affecting *trp* Operon Expression II

(1)**Tryptophan and IAA Present**; The antirepressor (IAA) competes with the co-repressor for the binding sites on the aporepressor. Binding of antirepressor does not produce the conformational change necessary to produce functional repressor and consequently transcription can occur.

(2)*Attenuation*; When tryptophan is abundant (A), the leader region (segment 1) of the *trp* mRNA is fully translated.Segment 2 interacts with the ribosome which enables segments 3 and 4 to base pair, signalling to the RNA polymerase to terminate transcription. When tryptophan is scarce (B), segment 3 and 4 do not interact because the ribosome is stalled at the *trp* codons of segment 1. Segment 2 interacts with segment 3 and consequently segments 3 and 4 cannot pair and transcription continues. Obtained from Biochemistry, Stryer 1981.

Figure 10:: Factors affecting *trp* operon expression

(1) Tryptophan and IAA present ::Competition

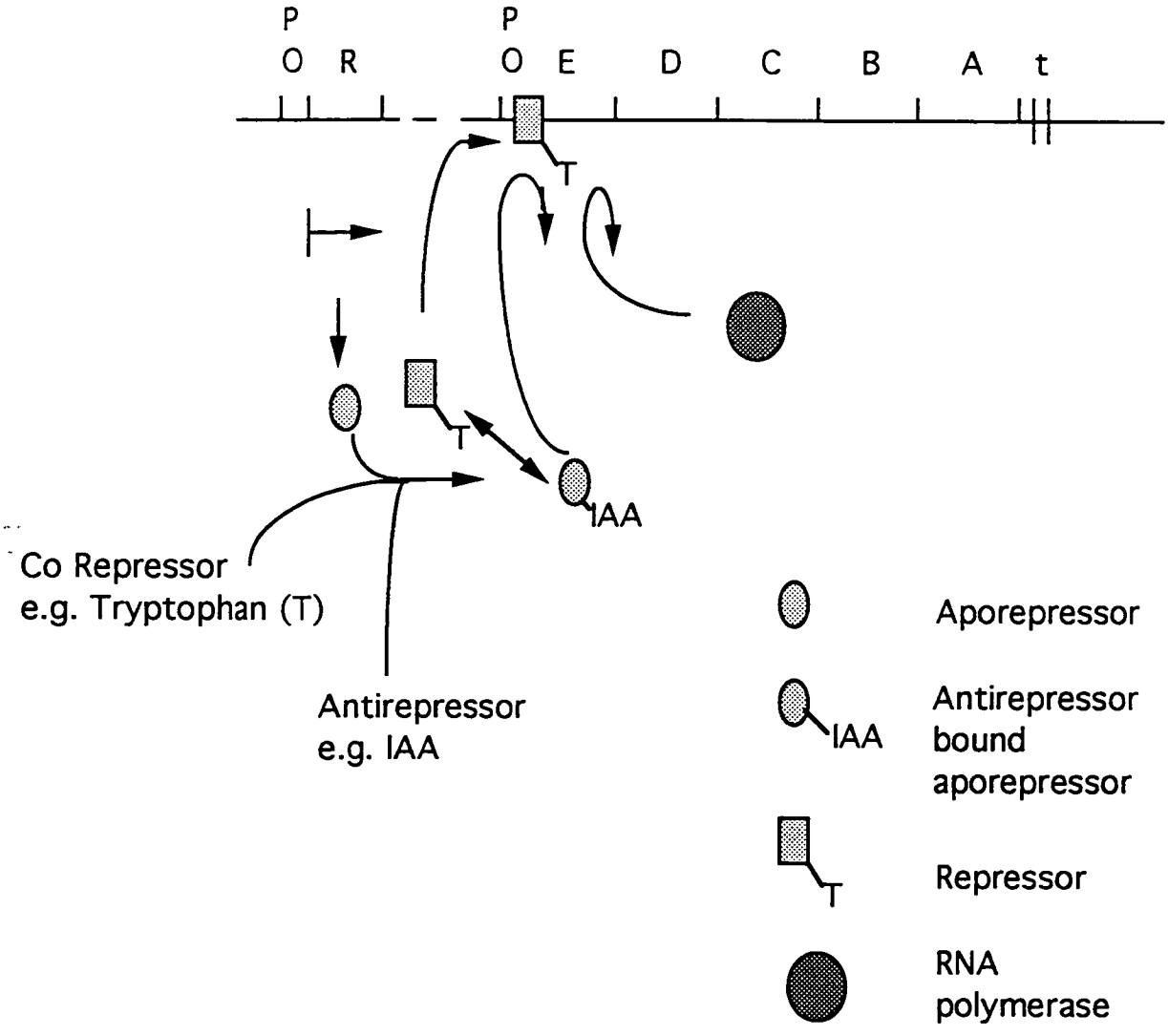
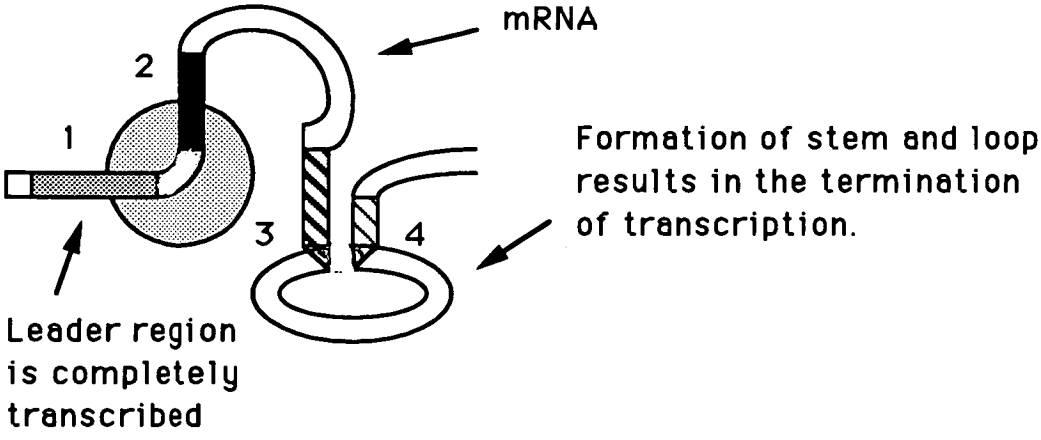
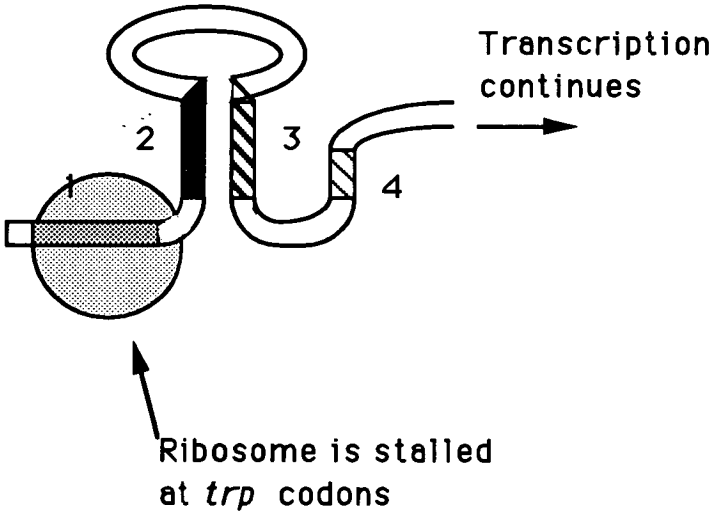


Figure 10 :: Factors affecting *trp* operon expression
(2) Attenuation

A. High tryptophan level



B. Low tryptophan level



4.3.1 Repression of *trp* Operon Expression

Within the promoter region (*trpP*) there is an operator site (*trpO*) at which regulation of transcription occurs [8, 97]. The *trp* aporepressor forms a complex with tryptophan which enables the resulting repressor, by some conformational change, to bind to the operator site [15]. This complex prevents the binding of RNA polymerase, and consequently eliminates transcription initiation, as shown in Figure 9.

A number of ligand binding studies have shown that a wide range of indole-containing molecules bind to the *trp* aporepressor with high affinity. Only molecules containing an amino group, however, can activate the aporepressor to form functional repressor [89]. The orientation of these tryptophan analogues on binding has also been demonstrated to be important i.e. the indole NH is required to point away from the interior of the protein toward the solvent [69, 89, 11] in order to form functional repressor.

Tryptophan analogues which do not fulfill these requirements e.g. 3-β-indole-acrylic acid (IAA) which has no amino group, but which can still bind to the aporepressor, act as "antirepressors" of the *trp* operon. These molecules compete with corepressors e.g. tryptophan, for the binding site on the *trp* repressor. Antirepressor binding does not result in a conformational change in the aporepressor and blocks the site for tryptophan. A means of establishing low internal pools of tryptophan should be used in conjunction with antirepres-

sors to ensure that the repressor complexes mainly with antirepressor. Since the TrpR-antirepressor complex cannot bind to the *trp* operator site, growth in the presence of antirepressor simulates tryptophan starvation conditions. RNA polymerase can bind to the operon under such conditions and transcription can occur. IAA was found to be a strong antirepressor of the *trp* operon [11].

4.3.2 Attenuation of *trp* Operon Expression

The rate of production of *trp* operon mRNA is also modulated by attenuation. Models of attenuation are based on the premise that one particular secondary structure signals transcription termination. If tryptophan is abundant, and therefore $tRNA^{trp}$ is available, translation of the leader peptide coding region will be closely coupled to transcription of the leader segment of the operon. The translating ribosome will prevent formation of the proximal secondary structure 2:3 permitting 3:4 to form, and thus resulting in termination, as shown in Figure 10b [105].

When the cell is starved of tryptophan, and $tRNA^{trp}$ is scarce, the translating ribosome will stall at one end of the tandem *trp* codons, preventing the formation of structure 3:4, and permitting alternate structure 2:3 to form. This will preclude formation of structure 3:4, and polymerase will transcribe through the attenuator site into the structural genes (Figure 10b) [105]. Those

RNA polymerase molecules that transcribe through the attenuator (which depends on the metabolic conditions) generally continue to the end of the operon.

4.3.3 Attenuation and Repression

Transcription of the 5 structural genes may therefore be regulated at both the operator and attenuator sites. When there are high levels of tryptophan, initiation of transcription is blocked by the binding of the *trp* repressor complex to the operator. As the level of tryptophan in the cell decreases, repression is lifted and transcription begins. Some of the RNA polymerase molecules will fall off the template at the attenuator site, whereas others will continue to synthesise the entire *trp* message. The proportion of RNA molecules that proceed past the attenuator site increases as the tryptophan concentration becomes lower.

4.3.4 Induction of *trp* Expression

When the chromosomally located *trp* operon is maximally expressed, the five *trp* polypeptides constitute greater than 10% of the total cellular protein. Thus, the chromosomal *trp* promoter can direct the synthesis of an average size protein to a level of approximately 2% of the cellular protein [84, 106].

Constitutive high level expression of a *trp* promoter can be achieved in *trp*R$^-$ strains which are unable to form the trp-repressor complex or where

the complex itself is unable to bind to the promoter DNA. When a cell is deprived of functional aporepressor, *trp* specific enzyme synthesis is induced about 70-fold above the basal/ repressed level [116].

In *E.coli* strains that contain functional aporepressors, a wide range of *trp* expression levels can be induced by the addition to the medium of a tryptophan antirepressor. A background level of tryptophan is required to prevent transcription stopping at the attenuator i.e. to ensure supply of charged $tRNA^{trp}$. A suitable concentration of the antirepressor can usually be found that will simulate a mild tryptophan deficiency, thereby fully derepressing transcription from the *trp* promoter [84].

Another means of obtaining a high level of *trp* operon expression is to use leaky *trp* auxotrophs. These strains have a mutation in one of the *trp* structural genes and produce an altered protein that is inefficient in tryptophan biosynthesis. The consequent reduced level of tryptophan results in a decreased level of functional repressor and also in transcription through the attenuator [84].

4.3.5 Method of Induction

Choice of a particular method of turning on a *trp* promoter depends on the aims of the experiment. If maximal overproduction is desired the tryptophan content of the protein to be expressed should be considered.

The major requirement for the work described in this thesis was control of expression over a wide range. For this reason the method of induction decided upon was the antirepressor, IAA. The mild tryptophan starvation conditions necessary to use IAA as an antirepressor complicate the system for the chemostat experiments, which require lactose as the only limiting nutrient. It was thought that by using a a *trp* auxotroph (*trp*$^-$ strain) in the chemostat, a reservoir concentration of tryptophan could be found which was low enough to allow induction but which would not affect the growth rate. If necessary the level of lactose would be adjusted, to maintain it as the limiting nutrient. Batch culture growth experiments with a *trp*$^-$ strain, NM107, have shown that the external tryptophan concentration can be reduced to very low levels before it starts to affect growth. Tryptophan was reduced to 0.5μg/ml before the initial growth rate was affected (S.Puri Personal Communication).

4.4 Subclones of the Tryptophan Promoter Region

Several subclones of *trp* promoter sequences were available, [116, 84, 106]. The *trp* promoter regions from *E.coli* and *S.marcescens* had been cloned into a series of plasmids by Yanofsky, to study expression of cloned genes. For the reasons given below the *S.marcescens* promoters were thought to be more appropriate for the requirements of this thesis.

4.4.1 The *S.marcescens* Promoter Region

The *S.marcescens* promoter is efficiently recognised by *E.coli* RNA polymerase [79]. In experiments which examined the function of the *S. marcescens*' attenuator, tryptophan starvation relieved transcription termination and the increase in operon expression was observed to exceed that typical of the *E.coli trp* operon [84]. The decision to use the *S. marcescens* promoter was based on its greater sensitivity and ability to express over a wider range than the *E.coli trp* promoter.

4.4.2 Plasmids containing the *S. marcescens trp* Promoter.

Nichols and Yanofsky subcloned five *S. marcescens* promoters with differing lengths of leader sequences, varying from no leader sequence to the complete leader sequences, into a pBR322 based plasmid [84]. The plasmid PBN37, which contains the complete promoter/attenuator region (Figure 11), gave the greatest range of expression with different levels of tryptophan in the medium and also contained restriction sites suitable for cloning downstream of the promoter. PBN37 also contained regions of the *E.coli trp* genes flanking the promoter, which would allow the possibility of homologous recombination as a method of integration of the cloned *lac* gene into the chromosome, as discussed in Chapters 1 and 4. Plasmid PBN37 was obtained from Nichols and Yanofsky.

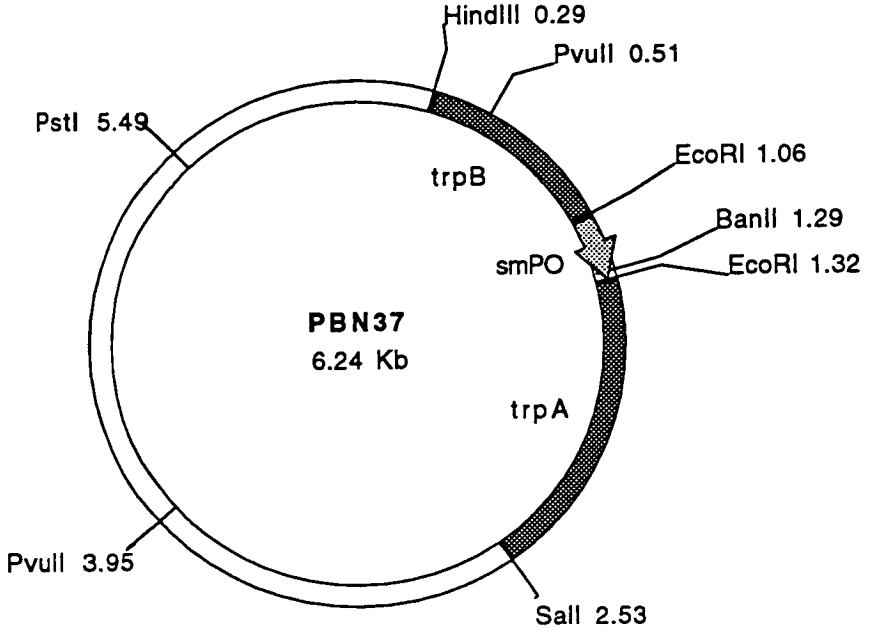
PBN37 (Figure 11) contains a HindIII-SalI fragment from the *E.coli trp*

operon, containing most of the *E.coli trpB* gene , all of the *E.coli trpA* gene and some sequence downstream of the *E.coli trpA* gene, including the termination site (*trpt*). The *S.marcesens* promoter (smPO) was inserted into the *trpB* gene.

Figure 11: Plasmid PBN37

PBN37 is a pBR322 based plasmid containing a HindIII-SalI fragment from the *E.coli trp* operon, incorporating most of the *E.coli trpB* gene, all of the *E.coli trpA* gene and the termination site *trpT*. The *S.marcescens trp* promoter (smPO) was inserted into a HpaI site within the *trpB* gene in an EcoRI fragment [84].

Figure 11:: Plasmid PBN37



4.4.3 Separation of the *lac* Genes

To choose which of the *lac* genes should be transferred to the control of the *trp* promoter the restriction maps of both the plasmid and the *lac* operon were examined carefully, using sequences obtained from the Wisconsin Database.

The decision to try and manipulate the *lacY* gene was based on its ease of manipulation and the availability of suitable *lac* mutants. Separating the *lacZ* gene from the *lac* promoter sequences, which was essential to ensure *lac*-independent induction of the gene, would not have been trivial. The complete *lacZ* gene was not obtainable in a single suitable restriction fragment.

A subclone was available which contained the entire *lacY* gene in a 2.1kb EcoRI fragment. This was generated by an *in vivo* deletion of the naturally occurring 18kb EcoRI fragment [5, 66]. The subclone, carried on plasmid pGB2601, had none of the *lac* promoter but contained the end of the *lacZ* gene and the complete *lacY* gene including its transcriptional stop signals [34, 43, 12]. This fragment also contained the complete sequence of the *lacA* gene and the upstream flanking sequences. Since the *lacA* gene does not have any known effect on growth, its inclusion in the construct was ignored. Functional *lac* permease had been expressed from this 2.1kb fragment in studies by Padan [93, 108, 12]. No other sequences were found necessary to achieve the correct insertion of *lac* permease into the membrane. The plasmid pGB2601 was obtained from Buechel *et al* [12].

A further consideration in the choice of the *lac* gene to clone behind the *trp* promoter was the content of the product encoded. The tryptophan content of the *lacY* gene product, plus the *lacA* gene product, was considerably lower than that of the *lacZ* gene product. The *lacZ* gene encodes a polypeptide which contains thirty six *trp* residues, whereas the *lacY* gene encodes only six *trp* residues and the *lacA* gene only two [53]. The effect of a tryptophan deficiency, as required for expression from the *trp* promoter by IAA, would therefore affect the yield of permease a great deal less than the β-galactosidase yield.

4.5 Subcloning the *lacY* Gene into PBN37.

The simplest approach was considered to be subcloning of the *lacY* gene, isolated from pGB2601 in the EcoRI fragment, into PBN37 at the EcoRI site downstream of the *S.marcescens* promoter. To achieve this a partial EcoRI digest was required so that only one of the EcoRI sites flanking the *trp* promoter was cut, and the promoter remained attached to the plasmid.

4.5.1 Preparation of Plasmid PBN37

The validity of plasmid PBN37 was checked with a number of restriction enzymes. An EcoRI digest was carried out to completion and the products were separated overnight on a 1.5% agarose gel. The small (258bp) EcoRI *S.marcescens* promoter fragment of PBN37 was identified by comparison of

mobility relative to 1kb molecular weight markers (Figure 12).

Conditions for partial restriction of PBN37 were optimised by setting up a series of reactions with differing reaction parameters. DNA concentration, enzyme concentration, type of restriction buffer and digestion time were varied. The digestion products were run on a 0.7% agarose gel to separate the DNA fragments. The reaction conditions which generated the largest amount of single cut plasmid were;

DNA (in TE)	10μl (10μg)
Restriction Buffer 10 x (BCL H)	5μl
Sterile dH_2O	34μl
EcoR1 (10 u/μl)	1μl

1 unit of EcoRI is the enzyme activity that completely cleaves 0.5μg of pBR322 DNA in 1hr at 37°C in a total volume of 50μl (1 x BCL H buffer).

The digest was incubated for 10-15mins at 37°C and stopped by adding 0.4μl 0.5M EDTA. The singly cut band of DNA was isolated as cleanly as possible from 0.7% agarose gels using NA45 paper, as described in the methods Section 3.3.1, and the concentration of DNA isolated was estimated on EtBr plates. The purified DNA was then used in ligation experiments with the *lacY* subclone.

The desired result was to have a large enough amount of singly cut plasmid to carry out the ligation experiments. The optimised reaction conditions did

not produce a large quantity of singly cut DNA as shown in Figure 13. Slowing the reaction down more had little or no affect on the efficiency of producing singly cut DNA since doubly cut fragments would always start to predominate after a certain amount of single cut had been produced. As the amount of DNA in both bands increased, the bands themselves began to merge on the gel. This made isolation of a clean, singly cut band difficult. From 10μg of undigested plasmid DNA it was typical to obtain 250-500ng of isolated singly cut DNA, the majority of DNA remaining undigested.

Figure 12: Complete EcoRI Digest of PBN37

PBN37	Lane 1	EcoRI
	Lane 2	1kb Markers

Figure 13: Partial Digestion of PBN37 with EcoRI

	Lane 1	1kb Markers
PBN37	Lane 2	Eco(p) 0mins
	Lane 3	Eco(p) 5mins
	Lane 4	Eco(p) 10mins
	Lane 5	Eco(p) 15mins
	Lane 6	Eco(p) 20mins

Figure 12

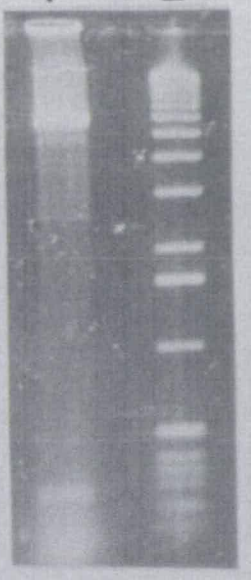
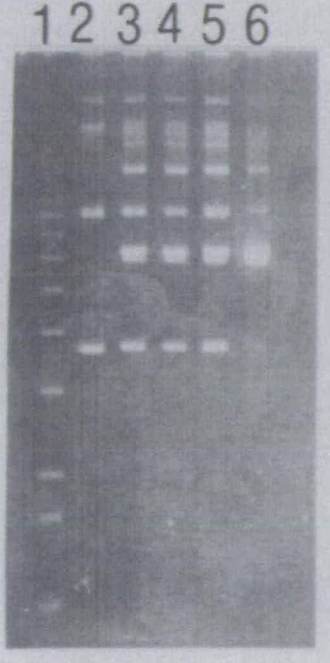


Figure 13



4.5.2 Preparation of *lacY* Gene Subclone.

The validity of plasmid pGB2601 was checked with a series of restriction digests. There was a discrepancy from the expected restriction pattern when the plasmid was digested with ClaI. The enzyme should have cut at sites within the ColE1 region of plasmid DNA and within the *lacY* fragment. However only one restriction site was found (Figure 14). On further examination of the plasmid using double digests it was apparent that the ClaI site was missing from within the *lacY* fragment.

An EcoRI digest allowed the 2.1kb EcoRI *lacY* containing fragment to be identified by comparison to molecular weight markers (Figure14).

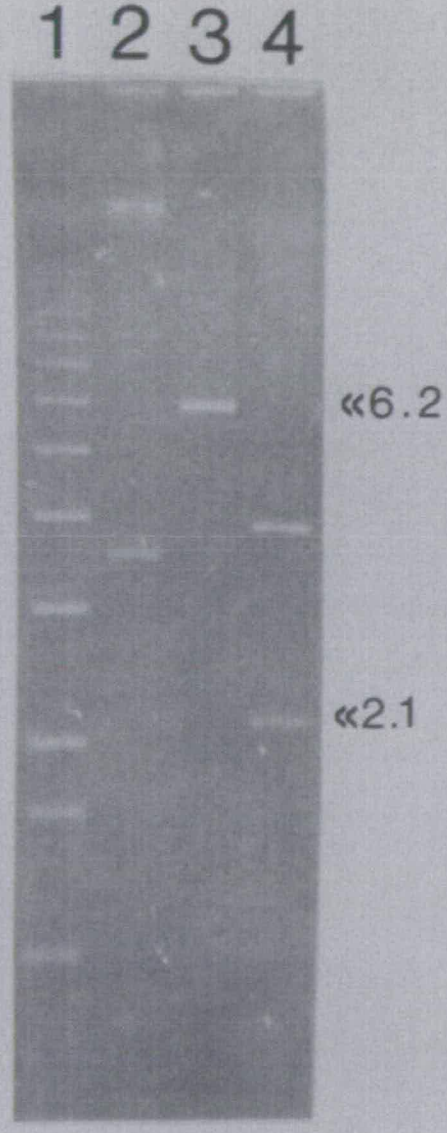
The 2.1kb restriction fragment was isolated from the gel using NA45 paper as described in the methods Section 3.3.1 and the concentration of the DNA was estimated by spotting on to EtBr plates with dilutions of control DNA of known concentration. The purified DNA fragment was used in ligation experiments with PBN37.

4.5.3 Ligation of *lacY* to PBN37

A series of ligation reactions were set up with varying the molar ratios of plasmid : insert in an attempt to obtain optimum subcloning conditions. The molar ratios used were 3:1; 1:1; 1:2; 1:3 plasmid : insert and were calculated as described in the methods Section 3.9.

The ligations were placed at room temperature overnight. (3μl) 10ng was then transformed into the *lacY*$^-$ strains, RE484 and RE478, using the Hanahan method described in Section 3.4.2, selecting only for amp^r in the first instance. Approximately 30ng of singly cut plasmid was used in each ligation. Transformants were screened for the presence of the *lacY* EcoRI fragment. The use of *lacY*$^-$ host strains allowed the success of subcloning to be checked by screening for functional *lac* permease.

Figure 14



4.6 Analysis of Transformants

4.6.1 Quick Screens for Functional *lac* Permease Expression.

The TONPG assay, described in Section 3.24.1 was proposed as a quick method of screening for functional permease within the population of transformants. This compound is a thio derivative of ONPG which is lethal to cells which are expressing permease. In the presence of high enough levels of permease, TONPG is sufficiently concentrated within the cell to inhibit growth. Growth inhibition is strongest with succinate or acetate as the carbon source [53].

The assay was standarised for permease under *lac* promoter control. The concentration of TONPG used was 10^{-2}M in Hobson's Salts (x1) with succinate as the carbon source. IPTG (10^{-3}M) was added to the plates and a range of *lac* mutants were tested for growth in the presence and absence of TONPG.

TONPG selection was applied to transformants to screen for expression of the *lacY* gene from the *S. marcescens trp* promoter. Induction from the promoter should rely on both tryptophan concentration in the cells and the concentration of the inducer, IAA. There were no positive controls available to determine the effect of TONPG on cells expressing permease under the control of the *trp* promoter. The actual concentration of IAA used (40μg/ml), was that which Nichols and Yanofsky used in their experiments to express cloned genes [84]. The other constituents of the selection medium were as standardised for permease expression under *lac* promoter control.

Another quick screening method attempted to detect permease expression was selection for the ability to grow on lactose in the presence of the inducer IAA. The concentration of IAA was the same as above in Hobson's Salts (x1) with lactose as the carbon source. The host strains used were *lacY*$^-$, therefore transformants expressing the *lac* permease should be positively selected.

There were potential problems with these selection systems in that it was not known at what level the permease gene would be expressed and, therefore, whether or not there would be enough permease activity to produce the desired phenotype. An additional problem was that although the strains used in these experiments, RE478 and RE484, were *lacY* mutants, they were not *trp* mutants and, therefore, the internal level of tryptophan could not be controlled externally.

400 amp^r colonies were picked from the RE478 plates and replica plated onto TONPG/IAA/AMP plates, to give a negative selection of *lacY*$^+$ colonies and onto LAC/IAA plates, to give a positive selection for the ability to grow on lactose.

The results of these selection steps gave 80 possible *lacY*$^+$ colonies from the 400 replica plated transformants. Plasmid DNA minipreps of these colonies were prepared as described in the methods Section 3.2.2 and the DNA was digested using a range of restriction enzymes to determine whether they contained the fragment and the orientation of insertion.

4.6.2 Restriction Analysis of Transformants

The orientation of the *lacY* fragment was determined from expectations based on the restriction enzyme data available. Enzymes useful for this purpose were those which cut both the *lacY* fragment and the plasmid infrequently so that the products were easily analysable. Those enzymes which cut the *lacY* fragment either near the beginning or near the end were particularly useful for checking the orientation of the fragment. BanII was the only enzyme which cut the *S.marcescens* promoter as well as the *lacY* DNA, this enzyme was important in determining the orientation of the *lacY* gene. Plasmid DNA from the transformants was digested with the same enzymes and compared with the different maps to determine the orientation of the *lacY* fragment.

The enzymes which were used in restriction mapping the fragment are listed in Table 6 along with the relative position of the restriction sites obtained from the DNA sequence databases available using GENBANK e.g. EMBL.

As an initial screen plasmid DNA minipreps of the 80 possible *lacY*$^+$ colonies identified from the TONPG assay (see Section 4.6.1) were prepared as described in Section 3.2.2 and the DNA was digested using EcoRI to check for the presence of 2.1kb fragment containing *lacY*; only 8 of 80 putative *lacY*$^+$ colonies had the desired fragment. Digestion with other restriction enzymes showed that none of the 8 had the *lacY* in the downstream site in the right orientation; 3 had lost the promoter altogether, 2 had the *lacY* in the upstream

site and 3 had the *lacY* fragment present in the downstream EcoRI site in the wrong orientation.

The 400 colonies were subsequently screened for *lacY* positives by colony hybridisation using the 2.1kb EcoRI fragment as a probe (Section 3.6.2).

The transformants to be screened were in the background of RE478 and RE474. These strains, although $lacY^{-}$, have the *E.coli lacY* gene present in the chromosome. This resulted in some background with the colony hybridisation but the high copy number (20-30x) of the plasmid, PBN37, ensured that the signal from the transformants containing plasmid with the *lacY* fragment was much stronger than that from the single genomic gene.

32 positives were found and plasmid miniprep DNA was prepared from those colonies not previously examined; None of the 32 positives had the *lacY* fragment in the downstream EcoRI site in the desired orientation. 18 of the positives had the *lacY* fragment but had lost the promoter; 4 had the *lacY* fragment in the downstream site but in the wrong orientation, 8 had the *lacY* fragment in the upstream site in a single orientation and 2 contained original plasmid, and thus were false positives.

It appeared surprising that none of the positives analysed had the *lacY* fragment in the correct orientation. However the six possible permutations were not obtained so this result may have simply been due to not examining enough *lacY* containing clones. 1 [93, 30, 36, 108] has shown that overexpression of the

permease under particular conditions is lethal to the cell. The conditions under which the present experiments were carried out were not similar to those of 1 but lethality was of concern. If permease expression was lethal under our conditions, there would be strong selection against the propagation of the desired construct. The small number of successful ligations achieved made it difficult to know if this was the case. However, no plasmids were found with only part of the *lacY* gene present which may also have been produced under such a scenario. Given the small number of positives analysed and the skew in the distribution of subcloned products, it was difficult to assess whether overexpression was a serious concern.

To increase the frequency of *lacY* subclones and the probability of obtaining the *lacY* fragment in the right site in the right orientation, the EcoRI site upstream of the *S.marcescens* promoter was destroyed.

Table 6: Restriction sites within Plasmid PBN37 and the *lacY* gene

Gene	Restriction Enzyme		
	BanII	EcoRI	PvuII
E.coli lacY	+627	+1, +2101	+13
E.coli trpB	-	-	+485
pBR322	-	-	+2608
S.marcescens PO	+226	+1, +258	-

4.7 Destruction of Upstream EcoRI site in PBN37.

An outline of the strategy for destruction of the EcoRI site is outlined in Figure 15. Singly cut plasmid was prepared and purified as described previously. The isolated fragment was then treated as described in Section 3.10 so that the overhang produced by the action of EcoRI restriction enzyme digest was filled in producing blunt ended DNA (Figure 15). This DNA was then ligated to eliminate the original EcoRI recognition site.

The singly cut band should have contained approximately 50% of molecules with the upstream site cut and 50% of molecules with the downstream site cut, giving a reasonable expectation of destroying the upstream EcoRI site.

After ligation the DNA was transformed into NM522, using a Hanahan transformation, and amp^r was selected for initially. Since there was no selection for the destruction of the site, plasmid miniprep DNA was prepared from the transformants and restriction digests were carried out to determine which, if any, of the EcoRI restriction sites had been destroyed. The restriction digests used were EcoRI; PstI; EcoRI/PstI double digest. The restriction patterns obtained were compared with untreated PBN37 digested with the same enzymes (Figure 16). Untreated PBN37 generates three restriction fragments when digested with EcoRI/PstI of sizes 4172bp, 1813bp and the 258bp *S.marcescens* promoter. If the upstream site of PBN37 was destroyed then the promoter fragment would be joined to the 1813 bp fragment to increase

its size to 2070bp. If the downstream site of PBN37 is destroyed then the promoter fragment would be joined to the 4172bp fragment to increase its size to 4429bp. In the plasmid PBN372 shown in Figure 16, the upstream EcoRI site has successfully been destroyed. The destruction of the upstream EcoRI site in PBN372 allowed the plasmid to be completely digested at the desired site without loss of the promoter fragment and therefore allowed efficient site-directed ligations of the *lacY* EcoRI fragment. To increase the efficiency of subcloning further the 5′ protruding ends of the EcoRI digested plasmid were phosphatased.

The dephosphorylation of the ends of the vector prevents plasmid religation to form original uncut plasmid. A complete EcoRI digest of PBN372 was precipitated and treated as described in Section 3.11. The phosphatased DNA was phenol extracted, resuspended in 1x ligation buffer, and ligated to *lacY* DNA as described in Section 3.9. The ligation mix was then transformed into strain G11 (*lac*Δ *Y*). 167 colonies were picked and screened for the presence of the *lac*Y^+ gene using colony hybridisation. 114 *lacY* positives were found. Plasmid DNA minipreps were made from each of the positives and a series of restriction digests were carried out to determine the orientation of the *lacY* fragment.

A plasmid with the *lacY* EcoRI fragment in the correct orientation, PBN372y, was found within the first 6 minipreps examined. The most conclusive evidence

that the *lacY* gene was in the correct orientation came from the BanII digest of pBN372y; a band of 656bp (Figure 17) was identified which confirms the restriction pattern expected from the sequence information. When probed with the EcoRI *S.marcescens* promoter fragment this 656bp band lights up showing, as expected, that part of the promoter is contained within this fragment (Table 6).

A restriction map of the constructed plasmid PBN372y is given in Figure 18.

Figure 15: Strategy of Destruction of Upstream EcoRI site in Plasmid PBN37

Plasmid PBN37 would be partially digested with the restriction enzyme EcoRI. The 5′ recessed ends would then be filled in using klenow to produce a blunt ended fragment. Ligation of blunt ended DNA fragment would result in the destruction of the EcoRI site. Restriction analysis of the end products of these reactions would determine which of the two possible EcoRI sites had been destroyed.

Figure15:: Strategy for destruction of Upstream EcoRI site in plasmid PBN37

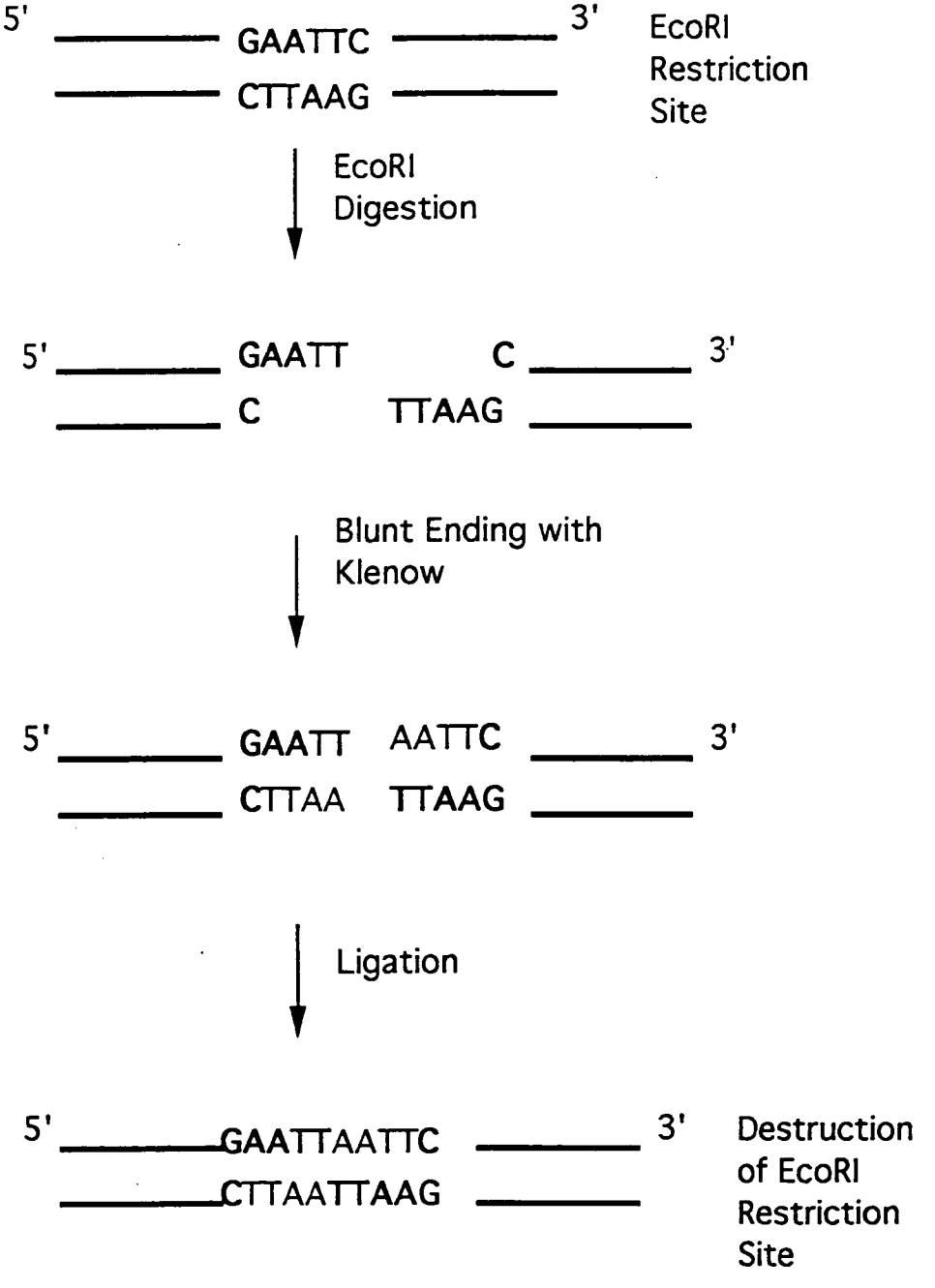


Figure 16: Products of Blunt end ligation of PBN37

(a)

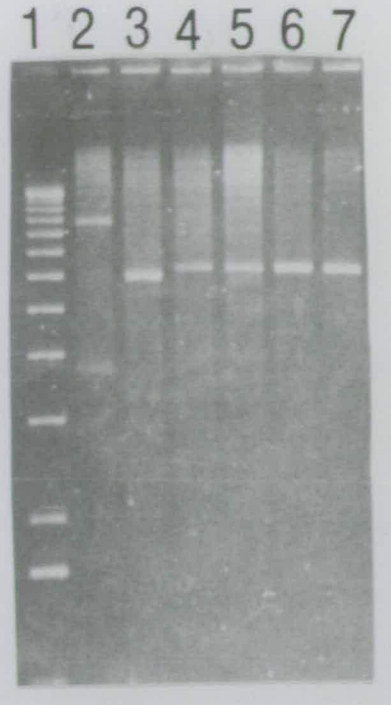
	Lane 1	1kb Markers
PBN37	Lane 2	Uncut
	Lane 3	EcoRI
PBN371	Lane 4	EcoRI
PBN372	Lane 5	EcoRI
PBN373	Lane 6	EcoRI
PBN37	Lane 7	PstI

(b)

PBN37	Lane 1	EcoRI
	Lane 2	PstI
PBN372	Lane 3	EcoRI/PstI
PBN37	Lane 4	EcoRI/PstI
PBN371	Lane 5	EcoRI/PstI

Figure 16

(a)



(b)

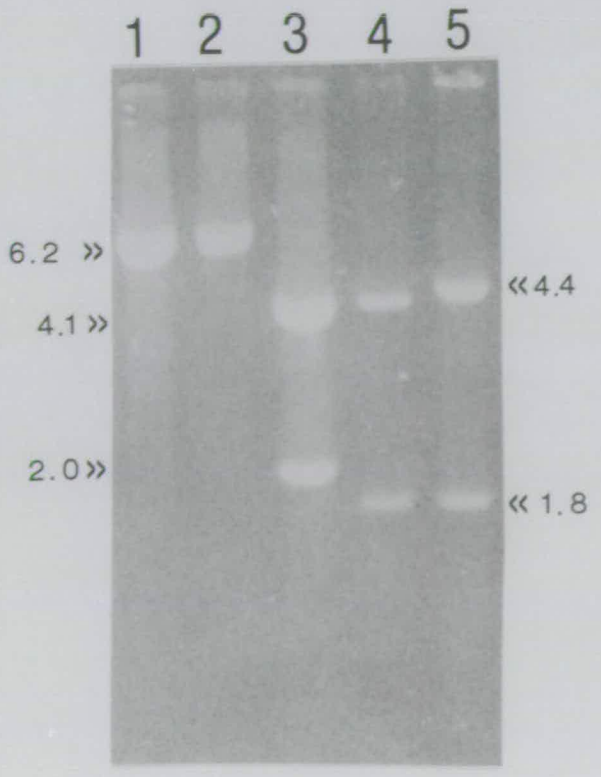


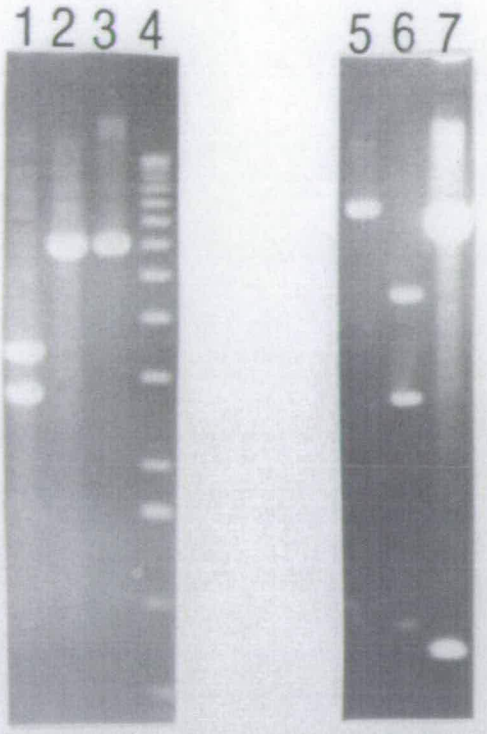
Figure 17: Restriction Digests of PBN372y

(a)		
PBN372	Lane 1	PvuII
	Lane 2	HpaI
	Lane 3	BanII
	Lane4	1kb Markers
PBN372y	Lane 5	HpaI
	Lane 6	PvuII
	Lane 7	BanII

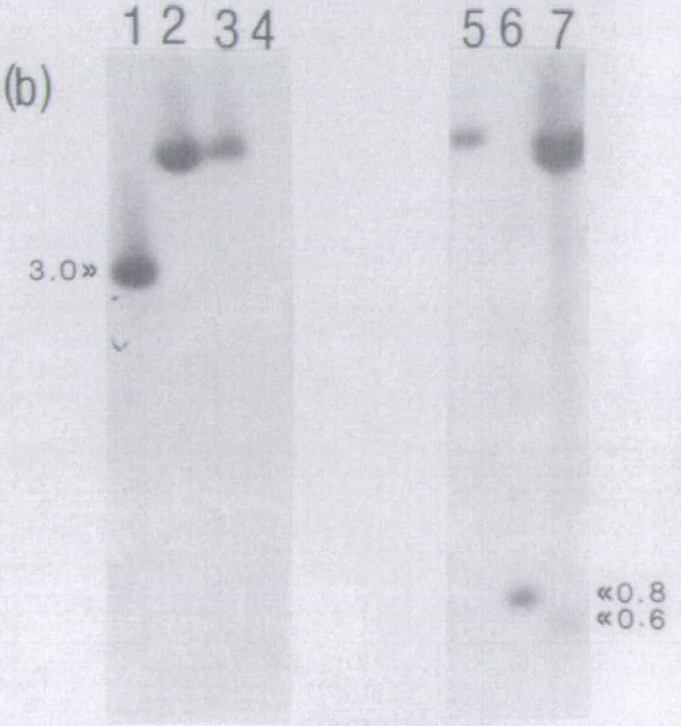
(b)smPO probe		
PBN372	Lane 1	PvuII
	Lane 2	HpaI
	Lane 3	BanII
	Lane 4	1kb Markers
PBN372y	Lane 5	HpaI
	Lane 6	PvuII
	Lane 7	BanII

Figure 17

(a)



(b)



4.8 Partial Sequence of PBN372y

The plasmid was partially sequenced over the *S.marcescens* promoter operator and *lacY* junction to confirm the construct was correct. An oligonucleotide was prepared by Oswel DNA service, based on a 21bp region of the *E.coli trpB* gene in plasmid PBN37 (1028-1048bp). The plasmid was sequenced from this primer using double-stranded DNA sequencing (Section 3.14), which prevented the need for subcloning from plasmid PBN372y. A large scale DNA preparation was used in the sequencing reactions (Section 3.2.1).

Partial sequence obtained from PBN372y (Figure 18) confirmed the destruction of the upstream EcoRI site and the correct insertion of *lacY* gene in the downstream EcoRI site. The initial sequence was extremely clear to read but from the attenuator site (+150bp of leader sequence) the sequence became more difficult to read. This was presumably due to the secondary structure around this area. This difficulty was overcome by altering some of the reaction parameters. The labelling mix was changed to the dITP labelling mix and the initial annealing reaction was changed to a slow annealing reaction. The annealing reaction was heated to 65°C for 3mins and then allowed to cool slowly to at least 30°C before the labelling reaction took place. These conditions allowed the attenuator site to be read through and the sequence obtained confirmed that the cloning had been successful.

Figure 18: Plasmid PBN372y

(a) A plasmid map of PBN372y highlighting the restriction enzymes used to determine the orientation of the *lacY* subclone.

(b) Partial sequence obtained at the junction of plasmid PBN372 and the *lacY* insert, highlighting the BanII site (1.29), EcoRI site (1.32) and PvuII site (1.36) shown in the plasmid map above.

Figure 18:: Plasmid PBN372y

(a) Restriction Map

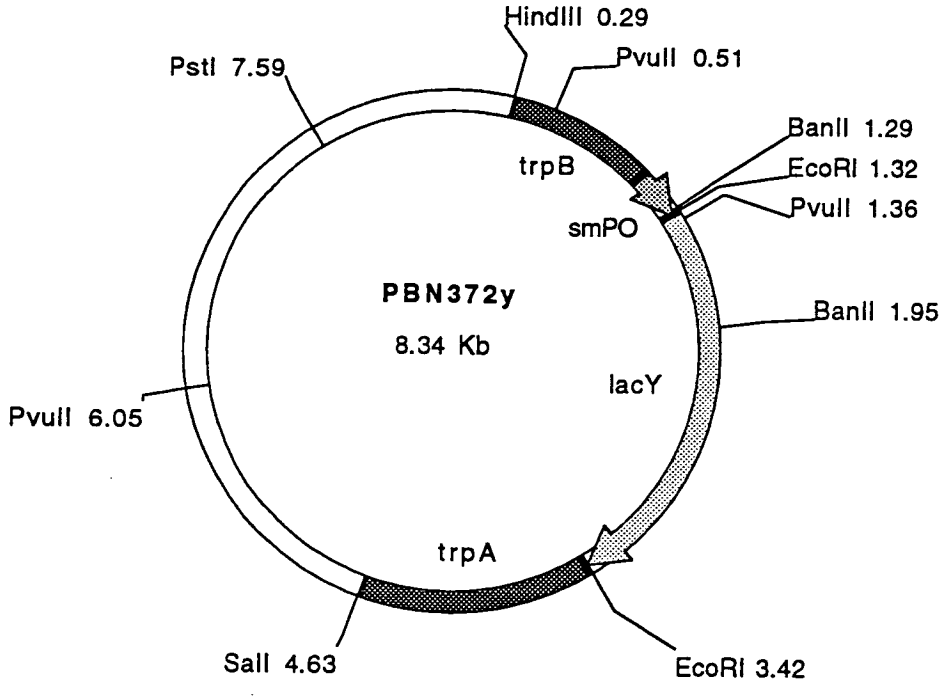
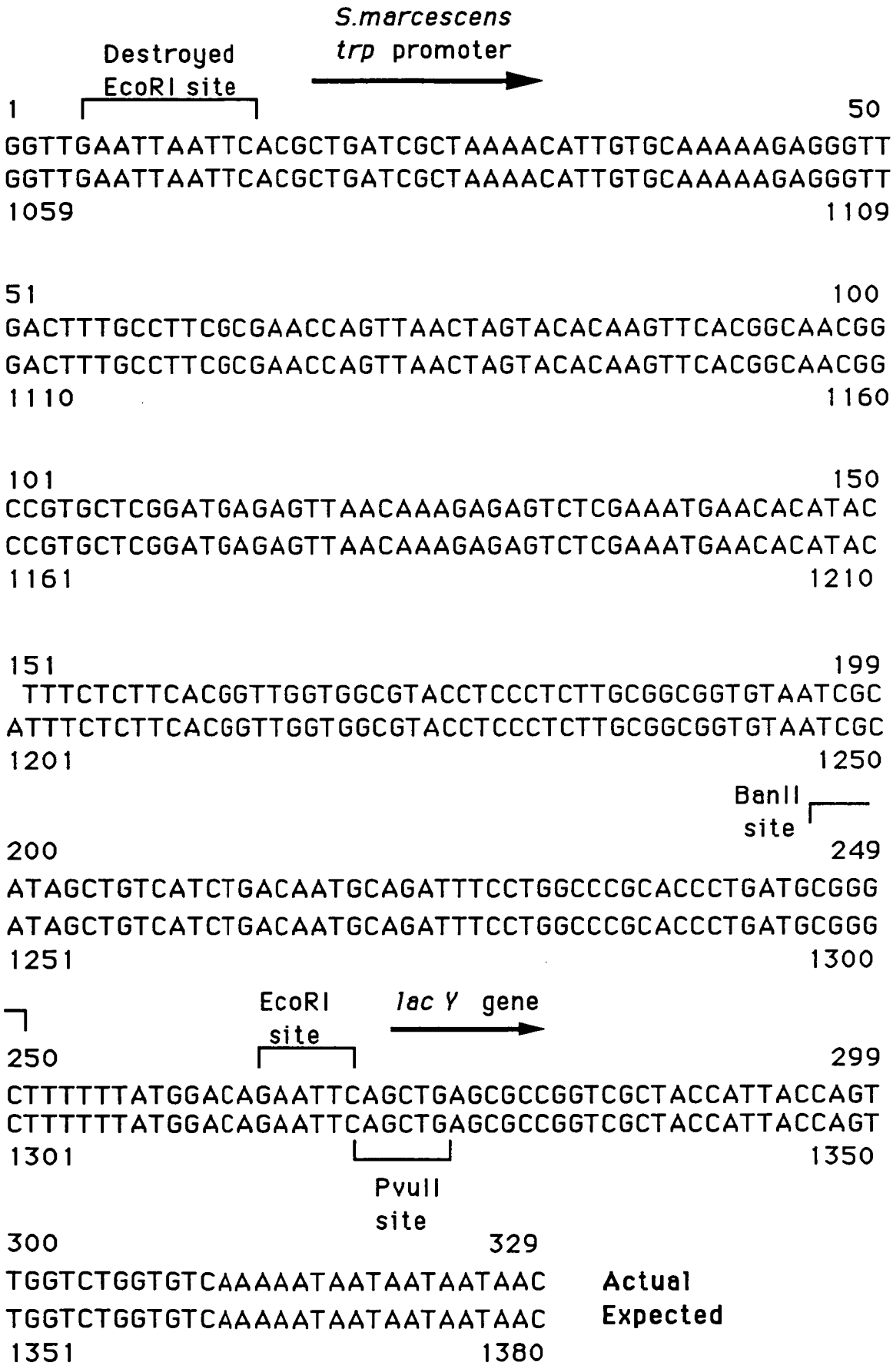


Figure 18(b):: Partial Sequence of the Junction between the *S.marcescens* promoter and the lacY insert



4.9 Assays for a functional *lac* Permease

The plasmid PBN372y, in the *lac*ΔY strain GII, was assayed for functional permease using the TONPG/IAA assay and Lactose/IAA assay. Controls were set up with a range of *lac* mutants and IPTG as the inducer. The different strains were replica plated onto the different media and their growth observed, see Table 7.

TONPG is lethal to cells producing large quantities of permease. G11 has a complete *lacY* deletion and therefore should be unaffected by TONPG in the presence of IPTG or IAA. The concentration of IAA (40μg/ml) used was observed to have a serious effect on the growth of strain G11 and the concentration was reduced to eliminate this effect. Table 7 shows the results of the optimised conditions, using 20μg/ml IAA and 6μg/ml TRP, the TONPG concentration was not changed. The reduced concentration of IAA was still observed to have a slight effect on the growth of strain G11 but the other control strains were unaffected. The addition of tryptophan to the medium allowed G11 to grow, apparently cancelling the effect of IAA. It was possible to conclude that the presence of TONPG/IAA is the cause of the reduction in growth in the strain containing the *lacY* construct (G11(PBN372y)) since the reduced IAA concentration had little effect. The observed growth of G11(PBN37) on TONPG/IAA would also suggest that the reduction in growth observed for G11(PBN372y) was due to high internal concentrations of TONPG and not

to IAA alone.

G11(PBN372y) showed limited growth on lactose minimal plates when induced with IAA, in the absence of tryptophan but grew well with tryptophan added to the medium (Table 8). This improvement in growth in the presence of tryptophan was again thought to be due to the negation of the deleterious effect of too high a concentration of IAA by tryptophan. Growth of strain 6947 appears to be unaffected by IAA. The very low level of growth of G11 (PBN372y) and NE4 observed on the LAC/IPTG plate was thought to be due to a low level of lactose diffusion through the membrane.

Table 7: Selection against *lacY*$^+$ using TONPG and IAA

Media	Strain					
	G11	G11(PBN372)	G11(PBN372y)	DD320	6947	NE4
Min(S)	++	++	+++	+++	+++	+++
IAA	+	++	++	+++	+++	++
TONPG/IPTG	++	++	++	+++	-	+++
TONPG/IPTG/TRP	++	++	++	+++	-	+++
TONPG/IAA	+	++	++	+++	-	+++
TONPG/IAA/TRP	+	++	++	++	-	+++

Table 8: Selection for ability to grow on Lactose with IAA v's tryptophan.

Media	Strain					
	G11	G11(PBN372)	G11(PBN372y)	DD320	6947	NE4
Min(L)	-	-	-	-	+++	-
LAC/IAA	-	-	++	-	+++	-
LAC/IAA/TRP	-	-	+++	-	+++	-
LAC/IPTG	-	-	+	-	+++	+

4.10 Discussion

The decision to clone the *lacY* gene was made because of the relative ease with which it could be separated from the *lac* promoter. Initial attempts to subclone *lacY* downstream of the *trp* promoter in PBN37 in the correct orientation for expression were unsuccessful. The possibility that overexpression of the permease from the *S.marcescens trp* promoter might be lethal to the cells containing the desired construct had to be considered. Since not all of the different possible permutations of *lacY* clones had been obtained it was decided that a much greater number of *lacY* clones needed to be isolated before any conclusions about overexpression could be drawn. The skew in the distribution of subclones suggested that it was a cloning problem only.

The initial ligation experiments were complicated by the presence of two EcoRI sites. The major EcoRI digestion product generated appeared to be double cut plasmid. It may have been possible to carry out the digest in the presence of varying concentrations of EtBr as a more efficient method of obtaining partial digests (Dr. I. S. Hunter, personal communication). The upstream EcoRI site of PBN37 was destroyed by klenow filling followed by blunt ended ligation. The elimination of this site increased the probability of cloning the fragment in the correct orientation to 1 in 2, as well as drastically increasing the efficiency of the ligation. If the subsequent ligation experiment had then produced *lacY* subclones in the wrong orientation only, it would have

indicated that overexpression was the problem. The actual results showed a greatly increased efficiency of subcloning and immediately generated the desired clone, PBN372y.

The TONPG expression assay was not used successfully as a quick screen for *lacY* expression and similarly the results of the LAC/IAA assay were not useful as a screen for functional permease. The lack of a positive control at this stage made the selection systems difficult to use with any confidence, since it was not known how much permease protein was being produced. Later results showed that the recommended amount of inducer IAA (40μg/ml) was not optimised to induce the permease to the level required to import TONPG in lethal quantities.

After successfully subcloning the *lacY* fragment, the above assays were carried out on strain G11 (*lac*Δ *Y*) containing the plasmid. The results were consistent with TONPG lethality after induction of permease expression with IAA. The concentrations of IAA and tryptophan used in these experiments, however, had to be altered from the concentrations stated in the original paper.

The *lacY* construct in PBN372y was next used to obtain a single stable copy of the *lacY* gene downstream of the *S.marcescens trp* promoter.

5 Chromosomal Integration Using a *recD* strain

5.1 Introduction

Having cloned the *lacY* gene downstream of the *S.marcescens trp* promoter and observed some expression of functional permease, it was essential, in order to carry out control analysis, that small changes in enzyme activity could be both induced and measured in the system to be studied. To do this accurately the induced enzyme activity must be the same from cell to cell within the population. With the *lacY* construct carried on a multicopy plasmid, the enzyme activity would vary from cell to cell with respect to the plasmid copy number.

5.2 Problems with plasmid instability

In high copy number plasmids e.g. pBR322 based plasmids, the copy number can vary from cell to cell. The copy number can range from 0-30 copies per cell and therefore has the capacity to vary enzyme activity, expressed on such a plasmid, considerably. PBN37 is pBR322 based [84, 7] and as such is subject to this type of variation in copy number.

or otherwise,

Cells, when in competition, may grow faster without plasmid [27, 68] or the selection may enrich for cells which have deleted parts of the plasmid, if the gene being expressed is not favourable to the cell. Consequently instability of plasmid in the chemostat may be a problem.

It was therefore important to have the construct stably maintained in the

cell such that the copy number was constant under all circumstances. This was best achieved by integrating the construct stably into the *E.coli* chromosome.

This Chapter outlines an attempt to integrate the *lacY* construct into the *E.coli* chromosome. In plasmid PBN372y the *lacY* construct is flanked by *E.coli trp* DNA. The flanking *trp* sequences should have allowed the integration of the *lacY* fragment into the chromosome as a result of homologous recombination with the *E.coli trp* operon. The *E.coli* strain made in this way would have the trp^- phenotype required for the controlled expression of the *lacY* gene from the *trp* promoter.

5.3 Homologous Recombination

Bacterial cells are generally haploid [102, 88], but homologous recombination can occur under a variety of circumstances. The cell may be made partially diploid by the introduction of DNA via conjugation, transduction or transformation. The DNA molecules available for recombination in these various circumstances may be circular or linear double-stranded or single stranded or a mixture of these. As long as there are homologous regions between the chromosome and the incoming DNA then recombination can occur.

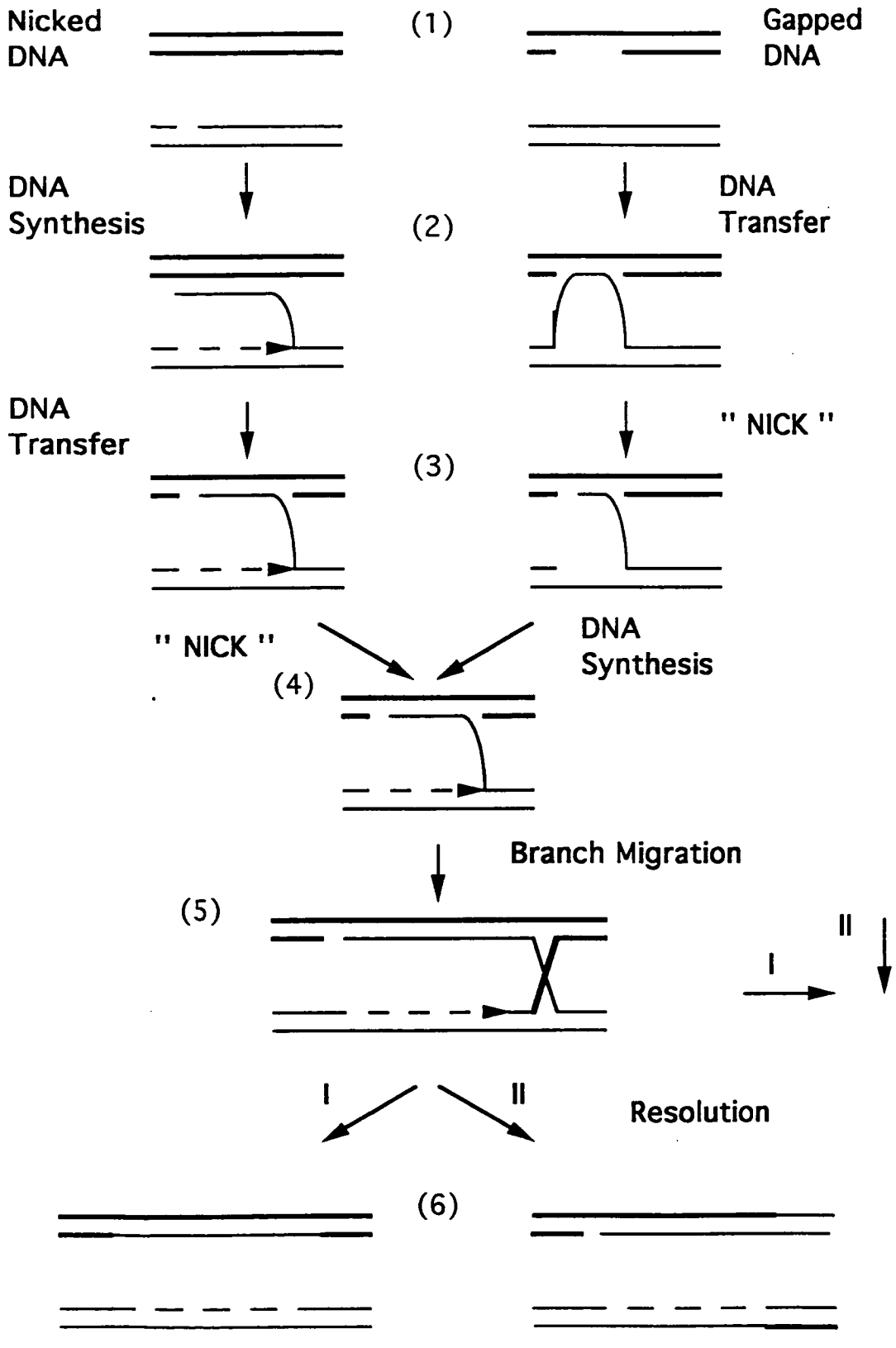
The mechanism of recombination, for each substrate, is thought to be similar [102].

5.3.1 A General Model Of Recombination

The initiation of recombination is thought to result from a discontinuity in the DNA i.e. a gap or a nick. The nick or gap produces a 3′-OH end of single stranded DNA (ssDNA) which invades a second double stranded (dsDNA) molecule to form a joint molecule or heteroduplex. This strand transfer reaction produces the central recombination intermediate proposed by Holliday [102, 110]. The crossover region can migrate along the region of homologous DNA by breaking base pairs at the crossover point and reforming them with the invading strand. This process of migration can enlarge the heteroduplex region and produce a second region of heteroduplex. The resolution of this structure into a recombinant DNA molecule (or two if the recombination is reciprocal) occurs by the introduction of strand breaks at the position of the crossover joint. The arrangement of these breaks determines the nature of the recombinants.

There are a number of different homologous recombination pathways in *E.coli*, involving different enzymatic reactions at one or more steps e.g. RecBCD, RecF and RecE pathways. The recombination pathway used is dependent on the nature of the substrate molecules available for recombination. The different recombination pathways all require the activity of the RecA protein.

Figure19 :: A Schematic Representation of General Recombination



5.3.2 The RecA protein

The RecA protein is a multifunctional enzyme [102, 28, 104] which catalyses the strand transfer reaction i.e. RecA catalyses the formation of the heteroduplex region in the recombination reaction described above.

Regions of non-homology can be created by RecA during this migration reaction, when the two DNA molecules contain differences in their sequence. It has been demonstrated that large regions of non-homology can be incorporated into the heteroduplex region [98].

Studies suggest that the RecBCD pathway is the major recombination pathway in wild type cells [102, 110].

5.3.3 The RecBCD pathway

The *recB*, *recC* and *recD* genes encode the subunits of exonuclease V, the RecBCD enzyme [102, 1, 110]. The RecBCD enzyme is the major nuclease for degrading DNA in *E.coli* and has been demonstrated to have ATP-dependent exonuclease activity and unwinding activities for dsDNA and, endonuclease and exonuclease activity for ssDNA, as well as a DNA dependent ATPase activity [1]. The enzyme does not act on covalently closed circular DNA or nicked circular duplex DNA but linear duplex DNA is the preferred substrate [102, 110]. Mutants of RecBCD pathway have been analysed with respect to these activities and it has been proposed that the unwinding activity generates

the ssDNA substrate required by RecA for heteroduplex formation.

Recombination via the RecBCD pathway is enhanced in the presence of *Chi* sites [102, 107, 110]. A *Chi* site has the nucleotide sequence 5′ GCTGGTGG 3′ and occurs approximately once in every 5kb of the *E.coli* chromosome. The effect is greatest close to the *Chi* site and decreases with increasing distance. *Chi* is not essential for recombination but may allow the pathway to function to its full potential. *In vitro* studies have shown that the RecBCD enzyme will cut duplex DNA containing a *Chi* site, on the strand containing the *Chi* site. The enhancement by *Chi* is thought to be as a result of nicking the duplex such that the DNA can then be unwound and used as the ssDNA substrate for recombination.

5.3.4 A Model for Recombination via the RecBCD pathway

The model of recombination proposed is described as follows [102, 110]: The *rec*BCD enzyme binds to the dsDNA end (1) and unwinds the DNA producing a loop-tail structure (2) and then a twin-loop structure (3). When the enzyme encounters a properly orientated *Chi* site, it cuts the strand containing it, converting one loop to two ssDNA tails (4). The enzyme continues to unwind, extending the 3′-OH tail containing the *Chi* near its end, and rewinds shortening the 5′ tail (5). Unwinding and rewinding continues until the enzyme releases the 5′ tail, resulting in the collapse of the second loop (6). At this

point, the enzyme has produced a long, possibly several kb, 3′ ssDNA tail extending from a dsDNA parental molecule. This long ssDNA molecule is used as a substrate for RecA and SSB and forms a D-loop (7). The displaced strand is transferred, by RecA, to fill the gap in the first parental strand. The hybrid DNA (hDNA), thus forms the Holliday structure which may be extended by the continued unwinding by the RecBCD enzyme and strand transfer by RecA.

Cutting of the Holliday junction, perhaps by the RecBCD enzyme and exchange of the DNA ends form either of two pairs of recombinants; resolution in the horizontal direction forms recombinants with hDNA flanked by parental DNA, whereas resolution in the vertical direction forms recombinants with hDNA flanked by recombinant DNA. After cutting the Holliday junction, any remaining gaps are filled in by DNA PolI and nicks are sealed by DNA ligase.

It was hoped that the flanking *E.coli trp* regions diagrammed in Figure 21 [110] would provide the homology for recombination by the mechanism described above, to generate a single chromosomal copy of the *lacY* gene under the *S.marcescens* promoter. Since the *lacY* gene interrupts the *trp* operon, the bacteria will be trp^-. The *trpB* and *trpA* genes are not reported to contain any *Chi* sites [3] and so it is unlikely that the recombination would be enhanced.

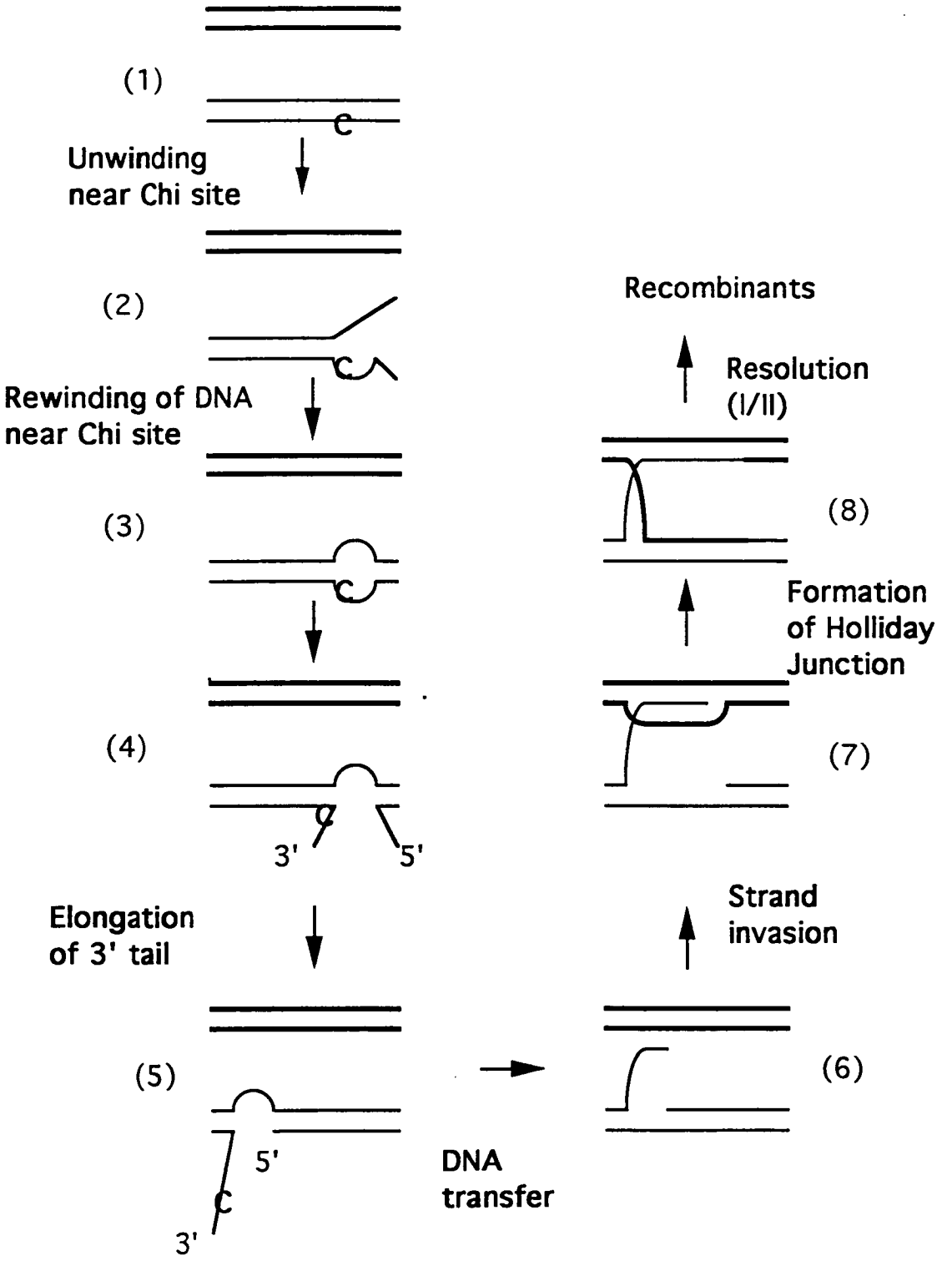
In order for the desired homologous recombination event to occur, however, the cloned DNA must be introduced into the cell successfully without being degraded [19]. Several approaches have been developed to allow the entry of

DNA into the cell and the subsequent homologous recombination event which integrates the foreign DNA into the *E.coli* chromosome. These approaches have had varying degrees of success.

Figure 20: Recombination via the RecBCD Pathway

A schematic representation of rearrangements of DNA during recombination via the RecBCD pathway [102, 110], where C represents a *Chi* site.

Figure 20 :: Schematic Representation of Recombination via the RecBCD pathway



5.4 Mechanisms of Integration

Mutations of the recombination pathways have been used to integrate specific genes into the *E.coli* chromosome. *recBC* mutants, in conjunction with *sbcBC* mutants allow the transformation of bacteria with linear DNA [19]. The absence of the RecBC enzyme reduces the efficiency of recombination to 1% of wild type. This deficiency can be suppressed by mutations in *sbcBC* which abolish exonuclease I or, in other strains, *sbcA* which leads to the synthesis of exonuclease VIII, the *recE* gene product. The restoration of recombination efficiency may in part be due to the susceptibility of linear duplex DNA to degradation by the RecBCD nuclease activity. Mutations inactivating the nuclease would provide increased stability of the linear substrate molecule. Strains of this type have been used in the study of *E.coli* chromosome recombination with plasmid DNA [112, 17].

Other methods employed involve selection for antibiotic sensitivity markers to select for plasmid integration [99] and then the subsequent selection for revertants which lose the resistance marker but retain the integrated gene [22]. This method was not chosen as it required further manipulation of the DNA construct since a resistance marker would have had to be introduced.

polA mutants have also been used to obtain integration of plasmids into the *E.coli* chromosome. The gene product of *polA*, DNA polymerase I, is required for plasmid replication. The only way a plasmid is maintained in a *polA*$^-$

strain, therefore, is to integrate into the chromosome [115, 55].

Those methods involving mutations, mentioned above, generally affect the growth of the host strain and were not desirable since this would complicate the chemostat experiments further. The other methods were not used because they required a selectable marker e.g. antibiotic resistance within the construct.

The method decided upon utilised a recombination pathway mutant which was reported to have no effect on growth and was recombination proficient [102, 99].

Figure 21: Homologous Recombination between the *E.coli trp* Operon and a Linear DNA Fragment

(a) A schematic representation of recombination of the chromosomal *E.coli trp* operon and a linear DNA fragment with some *trp* homology. Recombination results in chromosomal replacement of the wild type *trp* region with a mutant copy i.e. containing a *lacY* insertion.

(b) The expected restriction maps of both the wild type *E.coli trp* operon and the desired endproduct of the homologous recombination event, a *lacY* integrant.

Figure 21(a) :: A Schematic Representation of Homologous Recombination between the *E.coli trp* operon and a linear DNA fragment

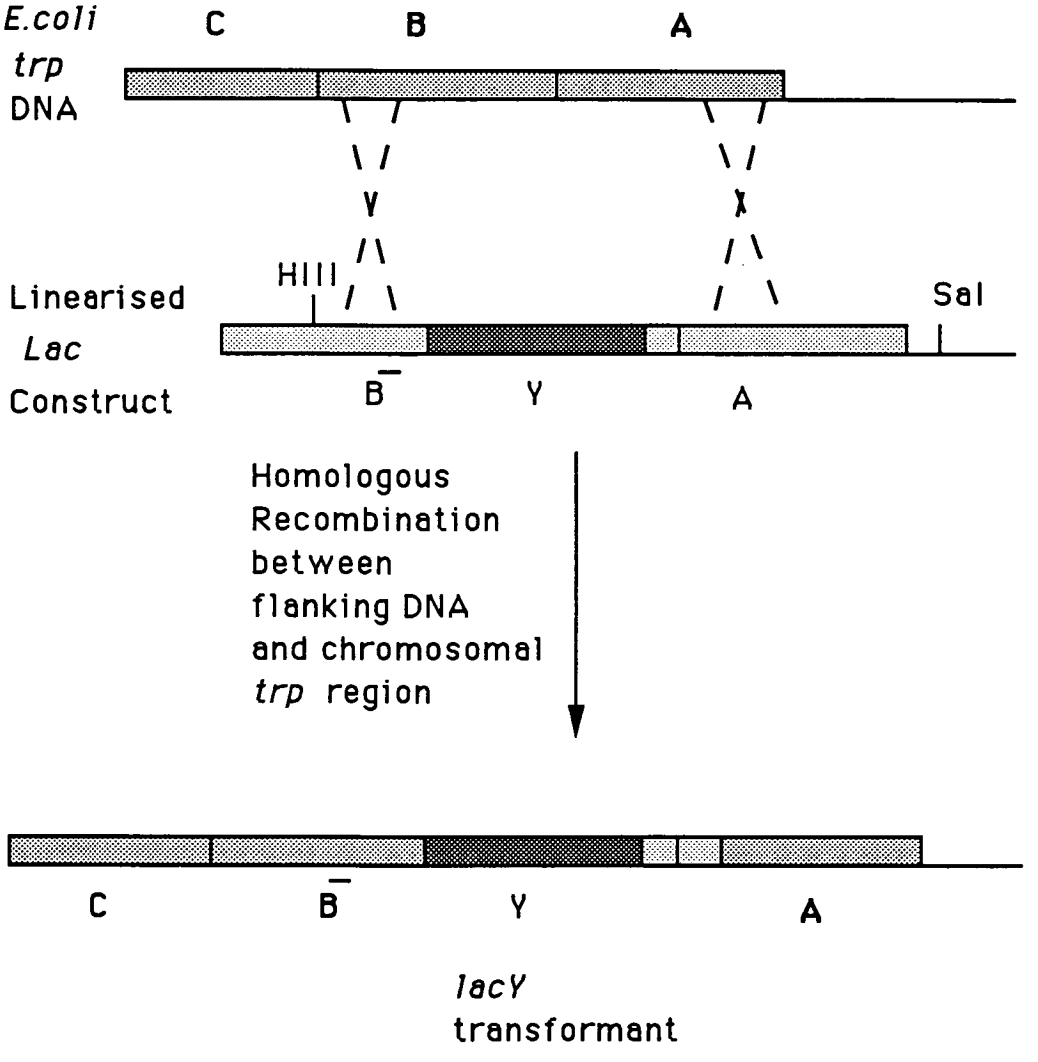
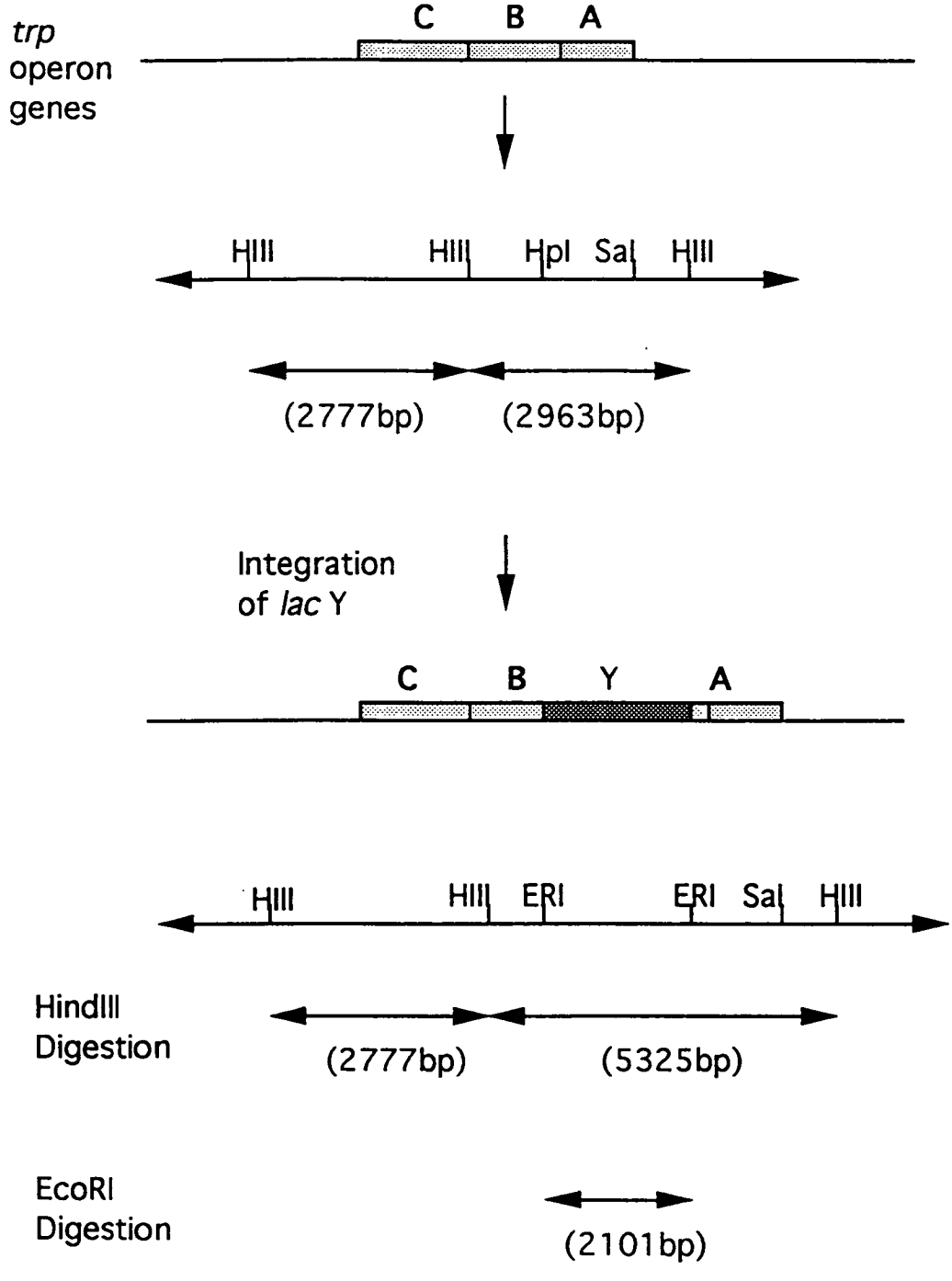


Figure 21(b):: The Restriction Map of the *E.coli trp* region before and after integration of the *lacY* contsruct



5.5 Chromosomal Transformation using a *recD* Mutant

Russell *et al* [99] examined chromosomal transformation using *recD* mutants. They have shown that *E.coli* containing a *recD* mutation could easily be transformed by linearised plasmids containing a selectable marker. The marker was transferred to the chromosome by homologous recombination whereas plasmid markers not in the region of homology were lost. This method was chosen because it appeared straightforward, requiring only a few genetic manipulations, and the efficiency of integration was high compared to other methods.

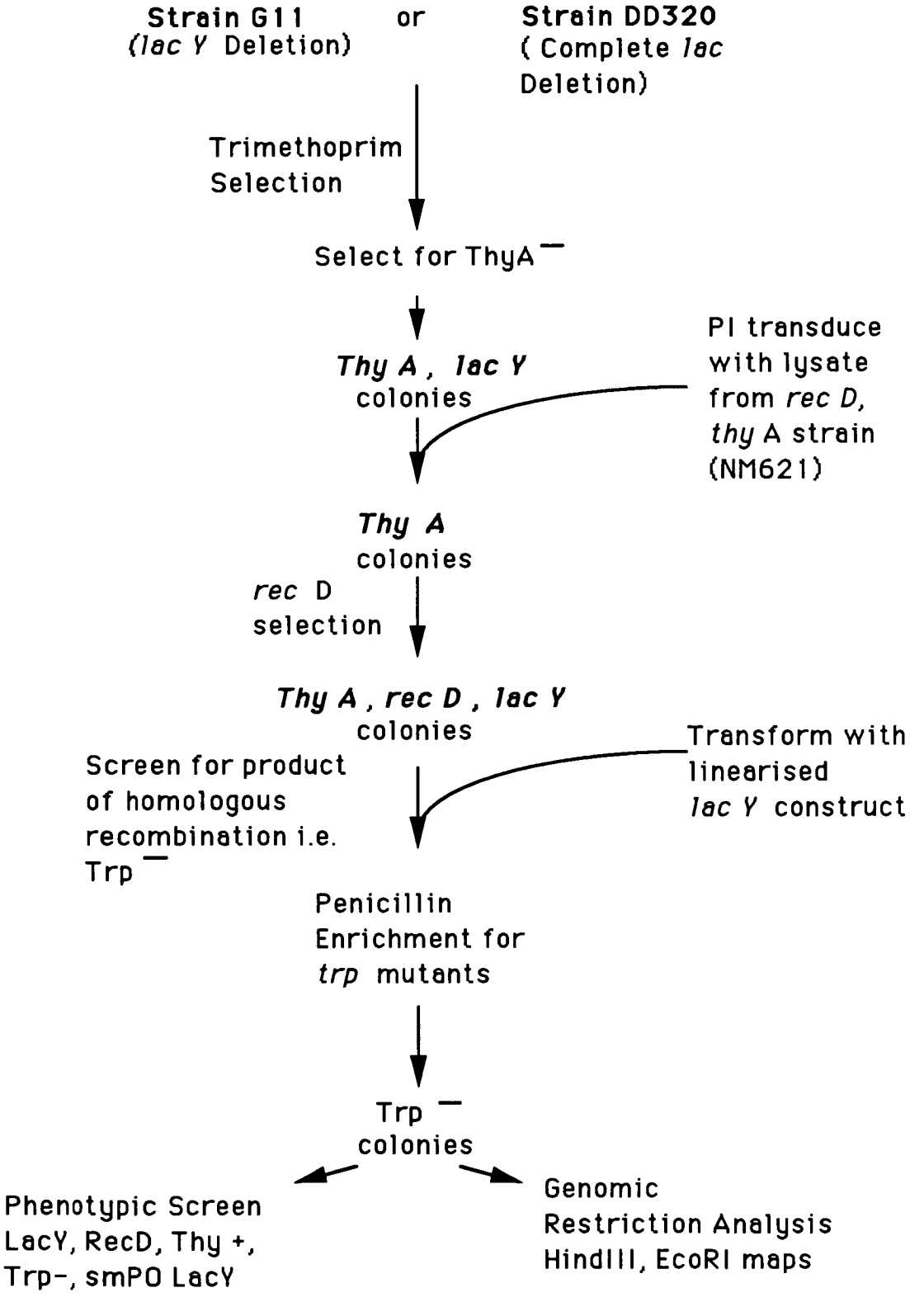
5.6 The *recD* Gene and Gene Product

The *recD* gene was isolated as a result of examining a class of mutants which lack the RecBCD nuclease activity but are recombination proficient. The *recD* mutants isolated were null mutations suggesting that the RecD protein is required for the nuclease activity [17]. The recombination proficiency of *recD* mutants is not understood. It has been hypothesised that these mutants retain the DNA unwinding activity of the RecBCD enzyme but this has not been successfully measured [102, 110].

The strategy, outlined in Figure 22, was to construct a recipient *recD* strain containing a *lacY* deletion. The *lacY* gene, under the control of the *S. marsescens* promoter and flanked by *E.coli trp* genes, would then be introduced into the *trp* operon. Homologous recombination between the *E.coli trp* regions

would be selected for by means of penicillin enrichment for Trp^- recombinants from the original Trp^+ recipient.

Figure 22:: Outline of Strategy to construct a *recD* recipient strain



5.7 Construction of *recD* mutant strain

Two strains with differing genetic backgrounds were used in this experiment, both of which were suitable as recipients for the *lacY* construct; DD320 contains a complete *lac* deletion in an otherwise wild type background; G11 contains a deletion of most of the *lacY* gene along with a *proC* mutation. The use of *lacY* deletion strains was important in order to prevent recombination between the *E.coli lac* region and the integrated *lacY* region at a later stage. Strain G11 was the preferred choice since only the *lacY* was deleted and no further manipulations would be necessary.

P1 lysates from a *recD* strain, NM621, were used, in a generalised transduction experiment to introduce the *recD* gene into the recipient strain. The transductants were then screened for the RecD phenotype. In order to provide a selection for transductants, a $thyA^-$ mutation was introduced into the recipient strain, the *thyA* locus being close to the *recD* locus on the *E.coli* chromosome.

5.7.1 Selection of *thyA* mutation in DD320 and G11

A simple method of producing *thyA* mutations in any strain of *E.coli* was available. Trimethoprim, with high concentrations of thymine or thymidine in the medium, is known to depress the growth of Thy^+ cells, but not that of Thy^- cells [54].

Both DD320 and G11 were passed through trimethoprim selection as described in the methods Section 3.15 [54], using the recommended trimethoprim concentration (200μg/ml). Strain G11 was unable to grow with this level of trimethoprim. Different sensitivities of strains to trimethoprim had been reported and in subsequent experiments the level for this strain was reduced to 50-100μg/ml. The resulting colonies were replica plated from LB + thymine plates onto minimal +/- thymine. The ThyA$^-$ colonies were selected and further tested for their *lac* phenotype and other markers diagnostic to the strain background. The frequency of production of *thyA*$^-$ mutants, for both strains is shown in Table 9.

The ThyA$^-$ strains were transduced to *thy*A$^+$ *recD*$^-$ using a P1 lysate of strain NM621.

Table 9: Trimethoprim selection of *ThyA*$^-$ mutants of Strains G11 and DD320

Strain	Minimal	Minimal+Thymine	% *ThyA*$^-$
G11	67	158	42%
DD320	110	161	68%

where % ThyA is the percentage of cells which do not grow on minimal medium compared with those which do grow on minimal medium.

5.7.2 P1 Transduction of *recD* Mutation into DD320 *thyA*$^-$ and G11 *thyA*$^-$

A P1 lysate of strain NM621 was prepared as described in the methods Section 3.13 [72, 48]. A P1 lysate can transduce a marker at a frequency of 10^{-4} to 10^{-5}, therefore it would be necessary to screen a large number of colonies, approximately 10^4 to 10^5, before isolating the specific marker desired. It is desirable therefore if there is some means of positively selecting the desired marker from the other transductants. The introduction of the *thyA* mutation into the recipient strain provides such a positive selection since *thyA* and *recD* are closely linked and are frequently included in the same P1 phage head. The *recD* *thyA*$^+$ strain, NM621, is *leu*$^-$, *pro*$^-$ and *thi*$^-$, allowing the initial selection of ThyA$^+$ transductants, on minimal glucose medium supplemented with proline, and the subsequent screening of these for the RecD phenotype, in either the DD320 or G11(*proC*$^-$) background.

Both DD320 and G11 were transduced with the lysate of NM621 as described in the methods Section 3.13 and the transductants plated out on glucose minimal medium supplemented with proline. The use of this medium immediately selects against colonies of strain NM621, eliminating contamination from the host strain, since this has other amino acid requirements not provided in the medium. This also eliminates growth of the recipient strains, both *thyA*$^-$, since thymine is not present. Thus, only transduction of the

region of the chromosome, from NM621, containing the *thyA* gene into the recipient strains can produce transductants able to grow on this medium.

The ThyA$^+$ transductants were then tested for the RecD phenotype.

5.7.3 Screen for RecD Phenotype

The ability to identify strains carrying the *recD* mutation relies upon the ability of λ *red*$^-$ *gam*$^-$ phage to form large plaques on *recD* strains but not on wild type bacterial lawns [102, 41, 110].

In phage λ replication, the concatemeric DNA to be packaged into the phage heads is made by two mechanisms (1) Rolling-circle replication and (2) Recombination. The concatemeric DNA synthesised by rolling-circle replication is successfully packaged to produce many phage particles. Recombination can produce dimers or multimeric circles which can act as a substrate for packaging. Rolling-circles are produced during the normal course of phage replication and dimeric circles are produced by the general recombination system of the *E.coli* host. In wild type *E.coli* this is generally the RecBCD system [102, 41].

Mutants in λ *red*$^-$*gam*$^-$ lack the phage encoded recombination system, the *red* system [110], and the inhibitor of RecBCD nuclease activity, the *gam* gene product [110]. These phage produce a reduced amount of concatemeric DNA and tend to make smaller plaques on *recBCD*$^+$ *E.coli* strains. λ*red*$^-$*gam*$^-$

phage , however, grow well on *recBC*, or *recD* mutants since these mutations allow the formation of rolling-circles by replication.

The phage used carries the deletion b1453 which removes the *red* and *gam* genes [41]. Thus, on *recBCD*$^+$ bacterial strains λb1453 makes very small plaques compared with wild type phage, however on *recD* strains the plaque sizes are comparable.

Dilutions of λb1453 were mixed with plating cells prepared from fresh cultures of the ThyA$^+$ transformants, and plated out in LB top agar and the plates incubated overnight at 37°C and examined for plaque size. Plating cells of a *recD*$^+$ strain, C600, and the original *recD*, NM621, were used as controls for the different strain backgrounds and λ220 was used as a wild type phage control.

16 ThyA$^+$ transductants of DD320 were picked and purified and 10 were found to *recD*. Unfortunately G11 apparently did not yield any *recD* co-transductants. The wild-type phage control also did not yield any plaques with strain G11 and it was concluded that strain G11 was resistant to phage λ and therefore the screen was unable to work with this strain.

The DD320 *recD* strain was used in the subsequent experiments. This strain contained a complete *lac* deletion and would therefore require the transduction of the *lacZ* gene from G11 at a later stage to complete the strain construction.

5.8 Introduction of Linearised DNA into *recD* Strain

Integration of linear DNA into the bacterial chromosome is slightly more efficient when both ends of the homologous region of DNA were free than if only one end was free [99, 110]. Therefore, plasmid PBN372y was linearised using restriction enzymes HindIII and SalI, generating a 4.6kb fragment containing the *lac*Y gene downstream of the *S. marcescens* promoter and flanked by *E.coli* *trp* genes B and A.

The fragment was separated by electrophoresis through a 1% gel and isolated using NA45 paper. The recipient strains were made competent using Hanahan transformation procedure and mixed with the linearised plasmid.

The anticipated homologous recombination event would generate a *trpB*$^-$ strain (Figure 21) and the transformants were therefore screened for Trp$^-$ bacteria.

5.9 Screening for *trp*$^-$ Transformants from a *trp*$^+$ Population

The efficiency of transformation reported by Russell *et al* for an antibiotic marker was 3.56 x 10^3 transformants/μg linearised DNA, from an initial 10^8 *E.coli* cells. Thus the frequency of transformation was 3.56 x 10^{-5}. Consequently for a simple screen for *trp*$^-$ colonies by replica plating, the number of colonies which would be required to be tested before the desired transformant would be likely to be found, would be greater than 10^5. It was thought nec-

essary to find some method of enriching for the *trp*$^-$ transformants. The best approach available was penicillin enrichment.

5.9.1 Penicillin Enrichment for *trp*$^-$ Transformants

This approach exploits the antibiotic, penicillin, which attacks growing cell walls, consequently, killing the cell [70, 54]. Auxotrophic cells can be enriched for using this antibiotic because in media lacking the specific requirement for growth the auxotrophic cells stop growing whereas the protrophic cells do not. Ampicillin can then be used to attack only the growing cells, killing them and allowing the auxotrophs to be rescued. The procedure is not completely selective, many prototrophic cells leak through. Penicillin enrichment has been reported to increase the frequency of desired auxotrophic mutations by 10^3 to 10^4 fold. Thus if the frequency was thought to be 1 in 10^5 in the transformation before enrichment, this treatment should reduce the screen to at least 100 penicillin survivors in order to find one colony with the desired mutation.

The method used was described in Miller [54]. An analogue of penicillin, ampicillin, was used in the experiments, since *E.coli* is more sensitive to this antibiotic and lower concentrations are required. The transformed cells were treated as described in the methods Section 3.16.

Of the resulting colonies screened (845) none was *trp*$^-$. The different stages of the experiment were analysed individually to increase the efficiency of se-

lection as much as possible.

5.9.2 Optimisation of Transformation of DD320*recD*

The transformation efficiency of the DD320*recD* strain was not high, the initial transformation efficiency being 7.6 x10^6 transformants/μg of uncut plasmid DNA. Control strains used in the same experiment transformed with a higher efficiency. Different methods of transformation were examined with DD320*recD*. The best transformation efficiency (5 x 10^7 transformants/μg) was obtained using a procedure described in the Promega protocols.

Repeating the transformation/ enrichment procedure with these competent cells yielded no *trp*$^-$ colonies in a screen of 950 ampicillin treatment survivors.

5.9.3 Optimisation of Penicillin Enrichment for *trp*$^-$ Mutants

The penicillin enrichment experiment was carried out as described in the methods Section 3.16 in a control experiment, using separate cultures of a Trp$^+$ strain, DD320*recD* and a Trp$^-$ strain, NM107.

The initial control experiment used 20μg/ml ampicillin for 60-90mins and lysis of the *trp*$^+$ control strain was not observed. The initial concentration of inoculum from both cultures was 10^7 cells/ml and the cells were treated with ampicillin and the number of viable cells monitored. The viable cell count of DD320*recD* was not observed to drop over the allotted time as shown in Figure

23. Thus, little enrichment was thought to have taken place.

Several experiments were then set up to allow a more intense selection.

A possible problem when increasing the selection is the simultaneous selection for penicillin resistant mutants during enrichment for a particular penicillin sensitive auxotroph. Resistant mutants are thought to be less of a problem when using ampicillin. This problem can be overcome, if necessary, by combining penicillin treatment with other enrichment procedures.

Initially however, only ampicillin concentration and the length of exposure to the antibiotic were varied. Separate cultures of the two control strains were set up to assess the affect of ampicillin on both trp^- and trp^+ cells. The time of exposure was increased in 30mins steps from 60mins to 180mins and the ampicillin concentration was increased from 25μg/ml in steps through to 500μg/ml. At each time point the cultures were sampled and dilutions were plated out on minimal and minimal supplemented with tryptophan, to monitor the viable cell count. 25 to 50 randomly picked colonies from each plate were replica plated onto minimal selection plates and tested for the original strain phenotype, no aberrant phenotypes were detected.

The optimum level was chosen at the point when the trp^+ strain lyses and the trp^- strain remains unaffected. The optimum conditions were found to be 200μg/ml for between 150-180 mins. The viable cell curve for both strains is shown in Figure 24.

After transformation of the DD320*recD* strain with 120 ng linearised DNA fragment, containing *lacY*, and two cycles of optimised penicillin enrichment, replica plating identified 75 possible Trp^- colonies from a screen of approximately 2000. These were reexamined and 62 were found to be Trp^- and lac^-. The cells were also tested for *amp* sensitivity and none of the Trp^- cells isolated was found to be resistant.

Figure 23: Penicillin Enrichment Control Experiment 1

The conditions recommended [54] were 25μg/ml ampicillin for 60-90mins, the control experiment showed no lysis during this period and therefore enrichment under these conditions was unlikely.

Figure 24: Penicillin Enrichment Control Experiment 2

The optimised conditions were 200μg/ml ampicillin for 150-180mins. After such a treatment the *trp*$^+$ control strain had shown some lysis while the *trp*$^-$ control strain remained unaffected.

Figure 23:: Penicllin Enrichment
Control Experiment 1

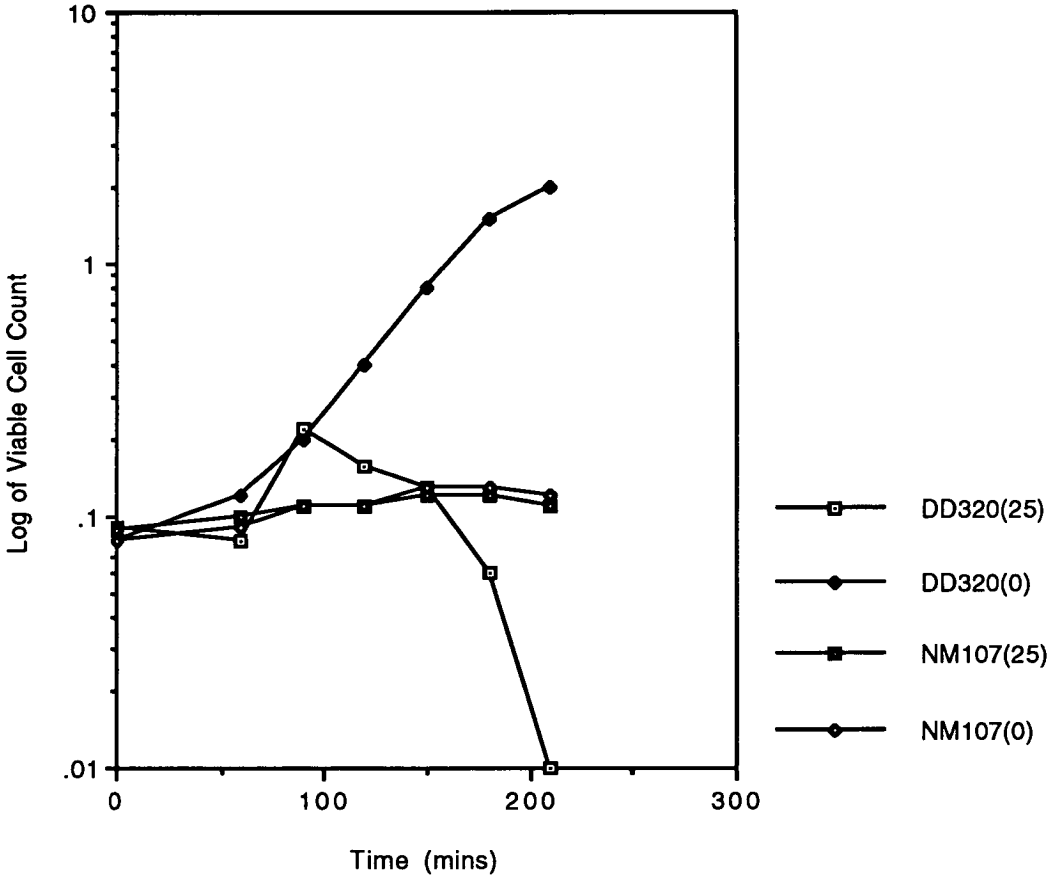
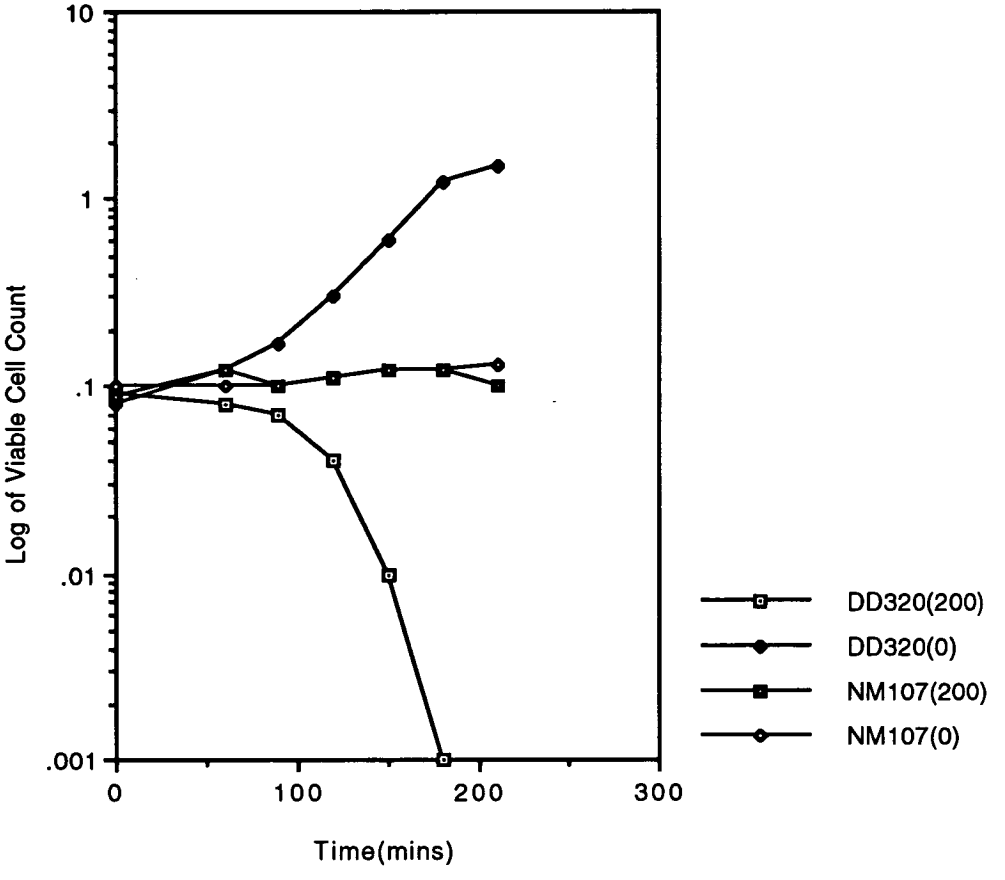


Figure 24:: Penecillin Enrichment Control Experiment 2



5.10 Analysis of *trp*$^-$ Mutants

5.10.1 Assays For Functional *lac* expression

Trp$^-$ colonies were examined for permease expression using TONPG/IAA plates.

No conclusive results were obtained. The concentration of tryptophan and IAA were varied independently in the medium, in an attempt to find optimum concentrations for expression of permease. The results did not suggest that the *lac* permease was being expressed since TONPG did not have any effect on the growth of the cells.

It was not possible to use LAC plates in the screen since the strain DD320*recD* contained a complete *lac* deletion and therefore was unable to produce any β-galactosidase irrespective of its permease status.

5.10.2 Restriction and Hybridisation Analysis

Colony blots were prepared as described in methods Section 3.6.2 and probed with the 2.1kb EcoRI fragment containing *lacY* and *lacA*. It was possible to use the 2.1kb fragment since DD320*recD* does not contain any *lac* sequences.

The colony hybridisation signals obtained were very poor. Comparison with controls showed a poor positive signal, which was thought to be due to only single genomic copy of *lacY* gene. 18 putative positives were picked and genomic DNA was prepared as described in the methods Section 3.1.

The genomic DNA was digested with the restriction enzyme EcoRI which would be expected, if the integration had been successful, to generate a 2.1kb band, when probed with the *lacY* fragment. This band is not normally present in wild type genomic DNA and an EcoRI digest would generate an 18kb fragment containing the *lacY* gene. Genomic DNA was prepared from a strain (NM107) wild type for the *lac* operon and digested with PvuII to generate a restriction fragment of approximately 2kb as a control for the hybridisation conditions (see Figure 18).

The DNA was probed with the 2.1kb EcoRI fragment containing the *lacY* which failed to hybridise to the genomic DNA from the trp^- colonies but did hybridise to a fragment of the correct size in the positive control track, shown in Figure 25.

HindIII digestion of the genomic DNA was expected to generate an altered restriction pattern in the trp^- integrants, no hybridisation signal was observed in these tracks when probed with the *lacY* EcoRI fragment.

It was therefore concluded that the trp^- phenotype did not result from integration of the trp^-smPO$lacY^+$ fragment.

Figure 25: Southern Analysis Of trp^- Genomic DNA

(a)Genomic DNA

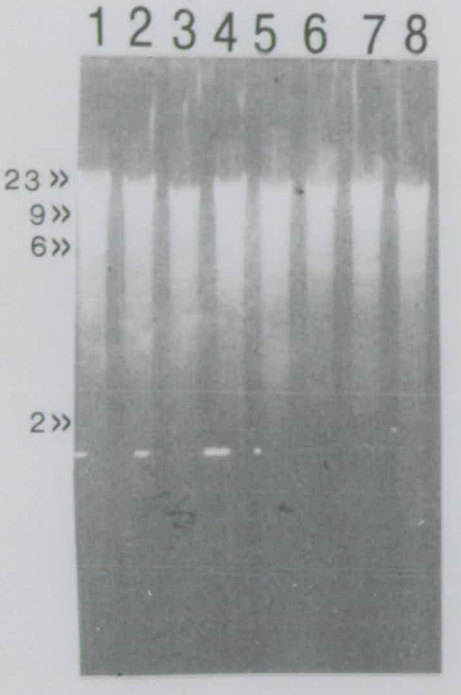
Lane 1	PvuII	NM107($lacY^+$, trp^-)
Lane 2	EcoRI	DD$recD$,Trp^- 1
Lane 3	EcoRI	DD$recD$,Trp^- 2
Lane 4	EcoRI	DD$recD$,Trp^- 3
Lane 5	EcoRI	DD$recD$,Trp^- 4
Lane 6	EcoRI	DD$recD$,Trp^- 5
Lane 7	EcoRI	DD$recD$,Trp^- 6
Lane 8	EcoRI	DD$recD$,Trp^- 7

(b)$lacY$ Probe

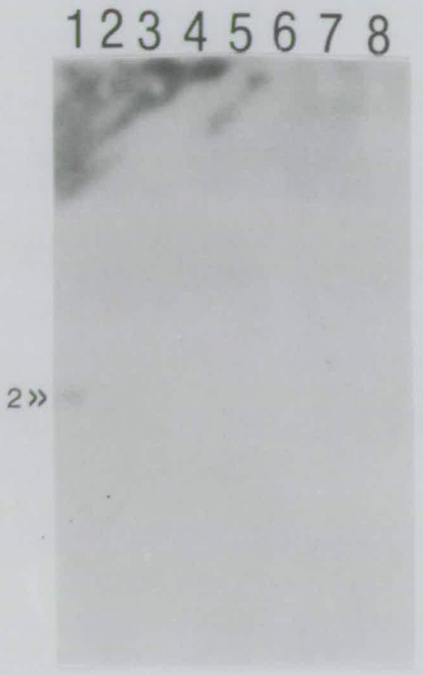
Lane 1	PvuII	NM107($lacY^+$, trp^-)
Lane 2	EcoRI	DD$recD$,Trp^- 1
Lane 3	EcoRI	DD$recD$,Trp^- 2
Lane 4	EcoRI	DD$recD$,Trp^- 3
Lane 5	EcoRI	DD$recD$,Trp^- 4
Lane 6	EcoRI	DD$recD$,Trp^- 5
Lane 7	EcoRI	DD$recD$,Trp^- 6
Lane 8	EcoRI	DD$recD$,Trp^- 7

Figure 25

(a)



(b)



5.11 Discussion

The point at which this experiment was unsuccessful was not clear, since the transformation efficiency was improved as well as the efficiency of selection. The penicillin enrichment, once optimised, resulted in the selection of spontaneous mutations only. The frequency of spontaneous mutations has been reported as 10^{-5} to 10^{-6} per generation for most loci in most organisms [31]. If the homolgous recombination/integration event was occurring at the expected frequency, *trp*$^-$ integrants should have also been isolated in the screen since they should have been occurring at an equal if not higher frequency compared with the spontaneous mutations. Indeed, this suggests that if the improved selection was enriching for spontaneous mutations 10^5 fold, integrants should also have been isolated in the screen. This would then suggest that the homologous recombination event was not occurring at the expected frequency.

The decision not to pursue this strategy further was based on the lack of confidence in both the selection system and the ability of the strain to form integrants.

An alternative integration strategy, previously dismissed because all prior cloning work had been carried out on multicopy plasmids and would have required further subcloning, was employed. This approach also had the advantage of not relying on a strong selection for the integration event.

6 Integration of *lacY* using Bacteriophage Lambda (λ)

6.1 Introduction

Due to the lack of success when using the *recD* strain to obtain *lacY* transformants, it was necessary to look for some other means of integrating the *lacY* construct into the *trp* region of the bacterial chromosome. Bacteriophage lambda (λ) has long been used for specialised transduction of bacterial DNA sequences [14].

6.2 Specialised Transduction

Genetic transduction is defined as the transfer of genetic information from a donor to a recipient cell by a virus. Phage λ naturally transduces only a limited and genetically linked set of host genes. A model for λ mediated specialised transduction has been proposed which suggests that the incorporation of host genes into a virus particle is the result of two recombination events. Initially the phage, as closed circular DNA, integrates into the chromosome to form a prophage (or lysogen). The crossover occurs at designated sites within the phage and the chromosomal DNA, *attP* and *attB* respectively, and the integration is catalysed by sequence specific phage and host proteins. Secondly, the phage is excised aberrantly, together with a segment of chromosomal DNA. This second recombination event has no sequence specificity and requires no known host or phage proteins. The natural substrate for packaging into the phage head is linear, concatenated phage genomes, produced by rolling circle

replication. The DNA is cleaved within the cohesive (*cos*) ends of the phage genome and packaged.

6.3 Specificity of Transduction

The specificity of the phage λ integration can be reduced by deleting the *attB* site on the chromosome. Phage λ has also been shown to integrate at a number of "secondary" attachment sites [110, 41]. Transducing phage unable to insert at an *attB* site usually will integrate where they have regions of homology with the host [110, 41]. Insertion by homologous recombination is promoted by the products of the *rec* genes. The efficiency increases with increasing homology but never equals normal wild type lambda insertion.

The production of such phages carrying any host gene has been made possible by the development of techniques for the directed transposition of selected genes.

This method normally entails subcloning mutated DNA fragments into a λ vector which encodes a temperature-sensitive repressor, e.g. λcI857, and selecting lysogens formed by crossing over between the phage-borne and chromosomal segments, and then selecting haploid mutant non-lysogens formed by a second crossover and loss of the excised prophage [14].

These properties of bacteriophage λ have been exploited further to integrate DNA fragments into specific sites in the chromosome, to develop a λ based method for moving mutant alleles into the bacterial chromosome directly from

plasmid vectors. Thus removing the need for the original subcloning step [67].

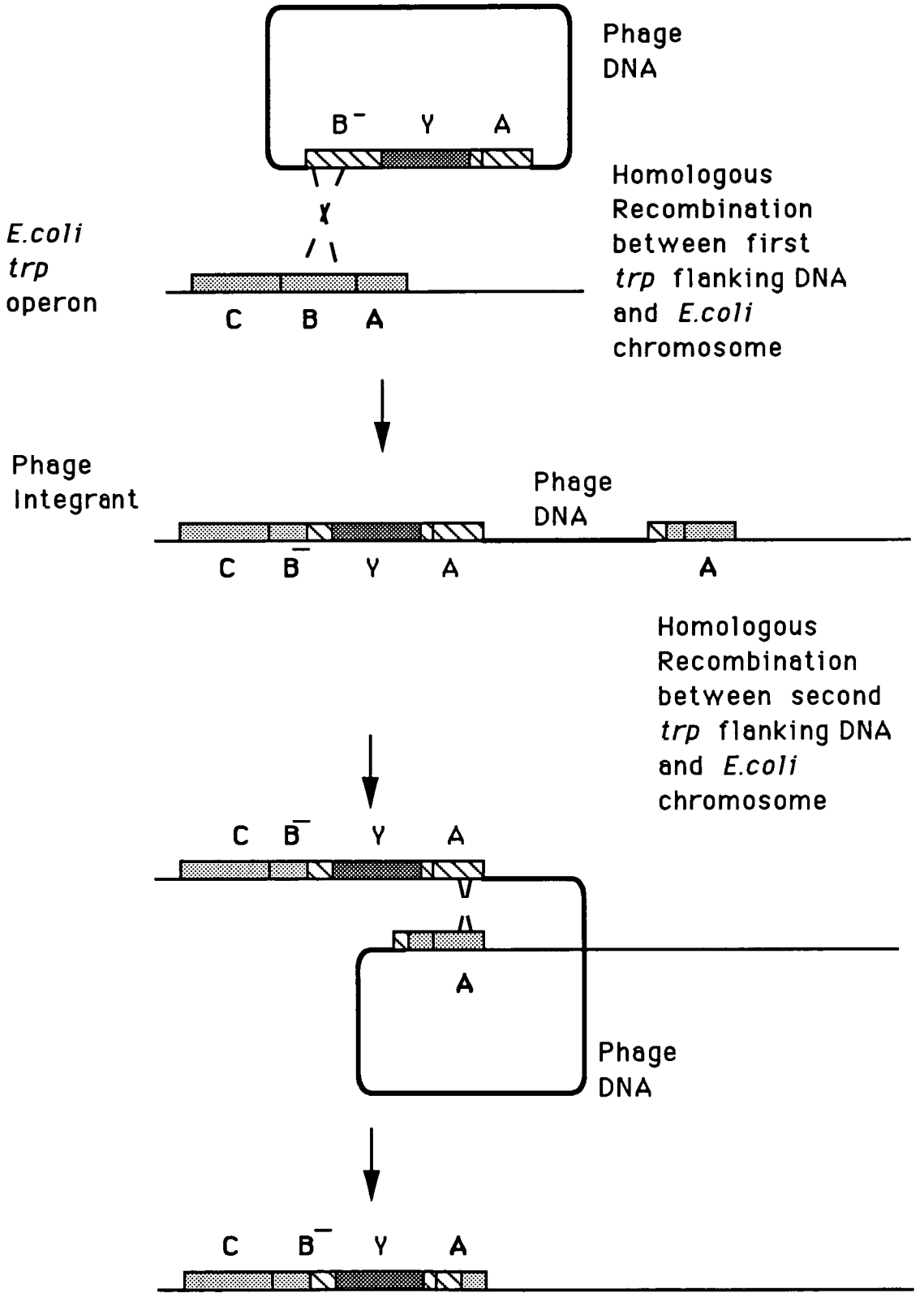
Kulakauskas *et al* [67] selected for antibiotic resistances to monitor transduction. The kanamycin resistance gene was inserted into the homologous region and the ampicillin resistance gene was plasmid borne. Cells carrying plasmids, with the homologous chromosomal sequences and the kan^r marker, were infected with phage λ containing cloned DNA, homologous to the chromosomal sequences. The resulting lysates were used for transduction. The λ phage containing cloned *E.coli* DNA are temperature sensitive *c*I mutants (repressor deficient at high temperatures) and thus normally kill sensitive bacteria above 37°C. Lysogens formed as a result of a single cross-over event could then be isolated at 32°C and, subsequent to a second recombination event, cured cells which retain the kan^r marker could be isolated.

It was hoped that λ transduction could be used to integrate the *lacY* construct as described in Figure 26.

Figure 26: Homologous Recombination between Phage λ DNA and *E.coli* Chromosomal DNA

A schematic representation of the possible DNA rearrangements involved in the homologous recombination of phage λ DNA carrying the *lacY* subclone and the *E.coli trp* operon. A single crossover event generates a phage integrant (prophage) containing the *lacY* construct. Resolution of this integrant can generate a wild type *trp* operon i.e. if the second recombination event occurs in the same flanking sequence (B) or a stably integrated copy of the *lacY* construct within the *trp* operon i.e. if the second recombination event occurs in the second flanking sequence (A).

Figure 26:: A Schematic Representation of Homologous Recombination between phage DNA and *E.coli* Chromosomal DNA



6.3.1 Effect of Strain Background on Transduction Efficiency.

The effect of different strain backgrounds on transduction efficiency was also investigated by Kulakauskas *et al* [67].

A several fold increase in the yield of chromosomal recombinants among transductants was obtained with a *dam*$^-$ (DNA adenine methylation deficient) strain. This may reflect replication deficiency of ColE1-type plasmids when transferred from *dam*$^+$ to *dam*$^-$ cells. Using bacteria with a *recD* mutation, which has been reported to affect plasmid instability, resulted in a ten fold increase in the frequency of transductants. The most dramatic result observed was obtained by using a *recBCsbcB* strain: all the recombinants were amp^s and plasmid free in both nonlysogen and lysogenic derivatives. The lack of plasmid transductants is probably due to an extreme instability of ColE1-type plasmids in this strain.

Suitable *recD* and *dam*$^-$ strains were already available i.e. DD320*recD* and NM547 respectively. A suitable *recBCsbcB* strain was not available and the construction of such a strain was not attempted.

6.4 Experimental Strategy For λ Specialised Transduction.

Two experiments were designed using this approach to transduce the *lacY* gene with *trp* flanking sequences, within phage λ, into the *trp* chromosomal genes of strain G11 (*lac*Δ *Y*). Both λ phage had varying amounts of *trp* homology and

used slightly different vector backgrounds to compensate for possible inviability of the phage. The strategy was to use the phage to obtain lysogens which can easily be selected for and then cure the cells, selecting for colonies which had phage integrated using the region of *trp* homology and subsequently undergone an excision event leaving the *lacY* construct behind in the chromosome, thereby conferring a TrpB$^-$ phenotype.

The phage were constructed as follows and are diagrammed in Figure 27;

(1)λ including a subclone of HindIII-SalI fragment of PBN372y, containing *lacY* flanked by part of *trpB* and all of *trpA*.

(2)λ containing the *trpABC* sequences with the *lacY* construct integrated into the *trpB* gene as a result of homologous recombination between the phage and plasmid PBN372y.

6.4.1 Construction of Phage 1

The phage designed in Experiment 1 was based on λ*c*I857 (48,502 bp) and contained a HindIII-SalI subclone of PBN372y (4.6kb). This fragment contains all the flanking *trp* sequences available in the plasmid and the *lacY* gene under the control of the *S.marcescens* promoter. The 4.6kb fragment was generated with two separate restriction digests using the required enzymes and their corresponding optimised restriction buffers and isolated from a 1% agarose gel as described in the methods Section 3.3.1.

Two separate restriction digests of λNM1 (λ*c*I857 *Sam7*) were carried out: SalI to generate the desired right (R) arm (15.769kb) from the restriction site at 32,745bp and HindIII to generate the desired left (L) arm (23.130kb) from the restriction site at 23,130bp. The total vector size would therefore be approximately 38.899kb and with insert 43.499kb which should allow successful packaging of the ligated phage.

The ligation was set up using the methods described in Section 3.9 with 500ng of vector DNA (2:3, right arm:left arm), and 200ng of insert. The ligation was packaged as described in methods Section 3.5 and dilutions of the packaging mix were plated out on the *E.coli* strain ED8654. Approximately 2,500 single plaques were probed with the *lacY* EcoRI fragment, as described in the methods Section 3.6.3 and 21 positives were picked and purified. A maxiprep of one of the positives was prepared as described in the methods Section 3.2.3 and restriction digests confirmed the presence of the *lacY* insert.

The phage was further tested using some genetic tests to identify the respective λ arms i.e. that the correct arms had been ligated. The phage should have been $trpA^+$, *Sam7* and $red^- gam^-$, as well as hybridising to *lacY*.

The phage were tested for $Trp^{+/-}$ phenotype as described in the methods Section 3.21 using λNM817 as a positive control. A halo of colonies were observed when the phage were spotted onto the $trpA^-$ strain (NM311) in the absence of tryptophan, suggesting the phage were $trpA^+$. No colonies were

observed on the control strains *trpC*$^-$ (NM127) and *trpB*$^-$ (NM312).

Strain NM675 (*supE*) was used to check the *Sam7* (right) arm was present, λ*c*I857 *Sam7* should not replicate as well on this strain since NM675 is *supF*. The control phages λNM122 (*red*$^-$, *gam*$^-$) and λNM123 (λ*c*I857 *Sam7*) would be expected to replicate well and badly respectively. Phage 1 grew equally on the *supE* background as λNM123 i.e. it was *Sam7*.

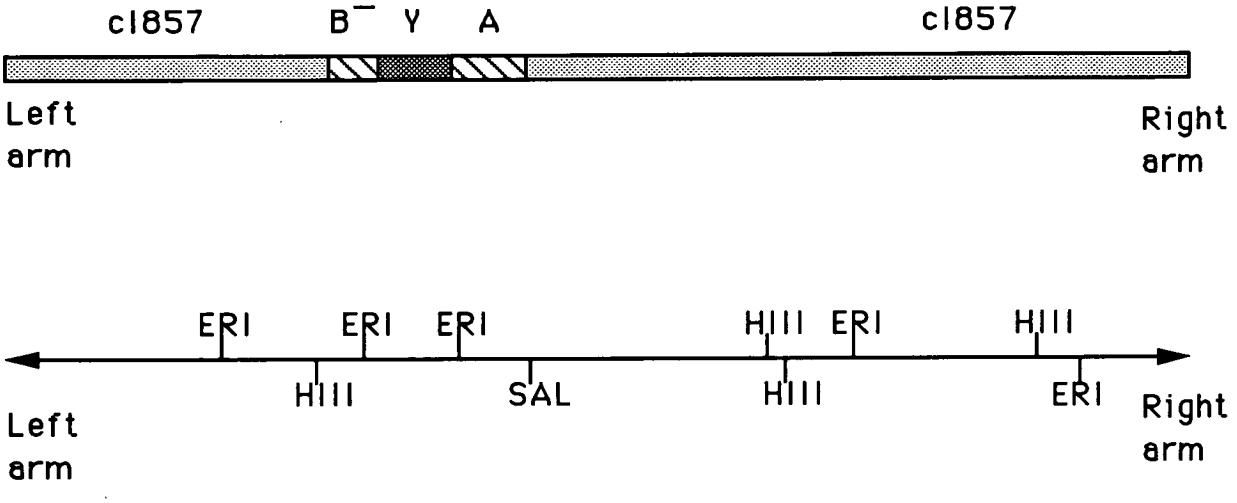
The *red*$^-$ *gam*$^-$ genotype was tested by plating on an *E.coli* strain carrying a P2 prophage, (NM539). λ*red*$^-$ *gam*$^-$ phage in contrast to λ wild type phage can form plaques on a *recBCD*$^+$ host lysogenic for phage P2. The newly constructed phage, like the λ*red*$^-$ *gam*$^-$ control (λNM1224), gave plaques on this strain and therefore appeared to have the correct right arm.

Figure 27: Phage λ Restriction Maps

(i) A schematic representation of Phage 1; the HindIII/SalI fragment of PBN372y, containing the *lacY* gene flanked by the *E.coli trp* genes *B* and *A*. The left and right arms were obtained from λNM1 using a HindIII digest and a SalI digest respectively. The expected restriction pattern of Phage 1 is shown, highlighting the HindIII/SalI subclone of the *lacY* construct and the expected restriction pattern for HindIII and EcoRI restriction fragments containing the *lacY* clone.

(ii) A schematic representation of Phage 2; the HindIII/SalI fragment of PBN372y, containing the *lacY* gene flanked by the *E.coli trp* genes *C,B* and *A*. The left and right arms were obtained from λNM1249 using a EcoRI digest and the *trpCBA* region was subcloned from λNM817. The expected restriction pattern of Phage 2 is shown, highlighting the HindIII/SalI subclone of the *lacY* construct and the expected restriction pattern for HindIII and EcoRI restriction fragments containing the *lacY* clone.

Figure 27(i) : Phage1 with PBN372y HindIII-SalI Fragment

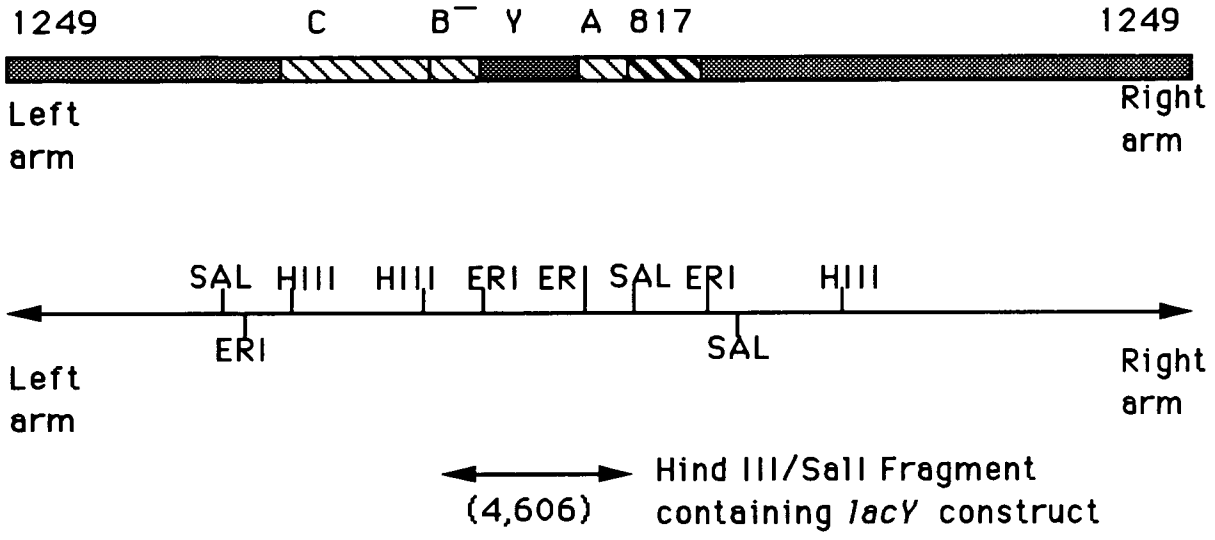


(4,606) Hind III/Sall Fragment containing *lacY* construct

HindIII Digest (8,252)

EcoRI Digest (2,101)

Figure 27(ii) : Phage2 with PBN372y HindIII-SalI Fragment



6.4.2 Construction of Phage 2

The phage designed in Experiment 2 was based on λNM1249, containing a subclone of *trpABC* in a 9kb fragment obtained from λNM817. Both phage NM1249 and NM817 were digested with restriction enzyme EcoRI and the ligation was set up as described in the methods Section 3.9 with 500ng of vector and 400ng of insert. The total vector size was calculated to be approximately 40.6kb which should allow for successful packaging of the phage and also the packaging of the final size of the phage after insertion of the *lacY* fragment, 42.7kb. The phage was packaged and dilutions of the packaging mix were plated out on minimal + ACH plates to select for Trp^+ plaques.

The trp^+ phage were plated, to confluent lysis, on two different strains of *E.coli* containing the plasmid PBN372y; a dam^- strain, 547 and a *recD* strain, DD320*recD*. A plate lysate was prepared, as described in the methods Section 3.12.2 from each of the host *E.coli* strains. The resulting lysates were titred and dilutions were plated out on ED8654, approximately 1,400 individual plaques were probed as described in the methods Section 3.6.3 with the *lacY* 2.1kb EcoRI fragment. 4 positives were picked, purified and reprobed. The resulting positives were screened for ampicillin resistance i.e. the presence of plasmid sequences.

None of the positives were found to be amp^r when tested, suggesting that integration had occurred and that the plasmid sequences had been lost. The

strain background did not appear to affect the frequency of recombination between plasmid and phage. Of the positives isolated 2 were found in the *recD* background and 2 were found in the *dam*$^-$ strain background. The different strain backgrounds did not generate significantly different results, a larger screen may have highlighted any effect strain background might have had [67, 110].

The phage were further tested using some genetic tests to identify the respective λ arms. The phage were expected to be *red*$^-$*gam*$^-$, *imm*λ rather than *imm*^{21} and *trpA*$^+$, *trpB*$^-$. The *red gam* and *trp* genotypes were tested as described in the previous section and the phage was found to be *trpA*$^+$, *trpB*$^-$ and *red*$^-$*gam*$^-$. The phage immunity was tested by plating the phage on different lysogenic strains; ED8654(*imm*λ), C600(λ*imm*^{21}) and NM621(λ*imm*^{21}). The phage were expected to be *imm*λ and therefore unable to grow on ED8654(*imm*λ). All the phage tested were *imm*λ.

6.5 Preparation of Lysogens of Strain G11 with Phage λ 1 and 2

The preferred background for the transduction experiments was the *lac*Δ *Y* strain, G11, since no further manipulations would be required subsequent to achieving the *lac Y* integration event. Strain G11 had previously been noted to be resistant to phage λ in Section 5.7.3. Since the mechanism of λ resistance in G11 was not known, the simplest method of constructing a λ sensitive strain

background for the *lac*Δ *Y* was to P1 transduce the mutation into a λ sensitive host.

6.5.1 Strategy for Transduction of *lac Y* Deletion from λ Resistant Strain (G11) to a suitable λ Sensitive Strain Background

A complex strategy was designed to move the *lac Y* construct to a λ sensitive strain which allowed selection at each step in the transduction and is outlined in Figure 28.

The desired background chosen was strain 614 which is prototrophic and λ sensitive. In order to transduce the *lac Y* deletion into this strain a transposon was used to tag the mutation to monitor its insertion by its antibiotic resistance, and then removed at the final step.

The transposon used was carried in strain DL615. The genotype of this strain was *phoR79*::Tn10 (*tet*^{r}) with *sbcC*$_{201}$ *proC*29 mutations close to the transposon insertion site (9mins) and *sbcB*^{15} *his*4 *arg*3 *recB*21 *recC*22 *galK* *mtl*1 *xyl*5 *supE rpsL*31 elsewhere. The transposon was situated within the *phoR* region of the chromosome which is within 1min of the *lac* region (8mins). P1 transduction can transfer a maximum of 100kb, equivalent to a genetic distance of 2min [5], and therefore the transposon could be co-transduced with *lac* region and used as a marker for the *lac*Δ *Y*.

A P1 lysate on DL615 was prepared as described in the methods Section

3.12.3 and used to transduce G11 to introduce the tag for the *lac*Δ *Y* (Step(1) Figure 28). Selection for transductants was made on minimal plates containing tetracycline and proline. The *tet*^{r} colonies were screened for *sbcC* cotransduction , *sbcC* co-tranduces with the transposon approximately 50% of the time, and *sbcC*$^+$ colonies were isolated [41].

A P1 lysate was then made using the *tet*^{r}, *sbcC*$^+$, *lac*Δ *Y* , *proC*$^-$ G11 strain and used to transduce the λ sensitive strain, NM614 (Step (4) Figure 28). Again the initial selection was carried out on tetracycline plates. The *tet*^{r} colonies were then screened for the *lac*Δ *Y* and *proC* mutations (Step (5)). Co-transduction of *lacY* and *proC* occurred at a frequency of 23%.

The *tet*^{r} colonies were also screened to check their sensitivity to λ. All of those tested were sensitive to a range of different λ phages.

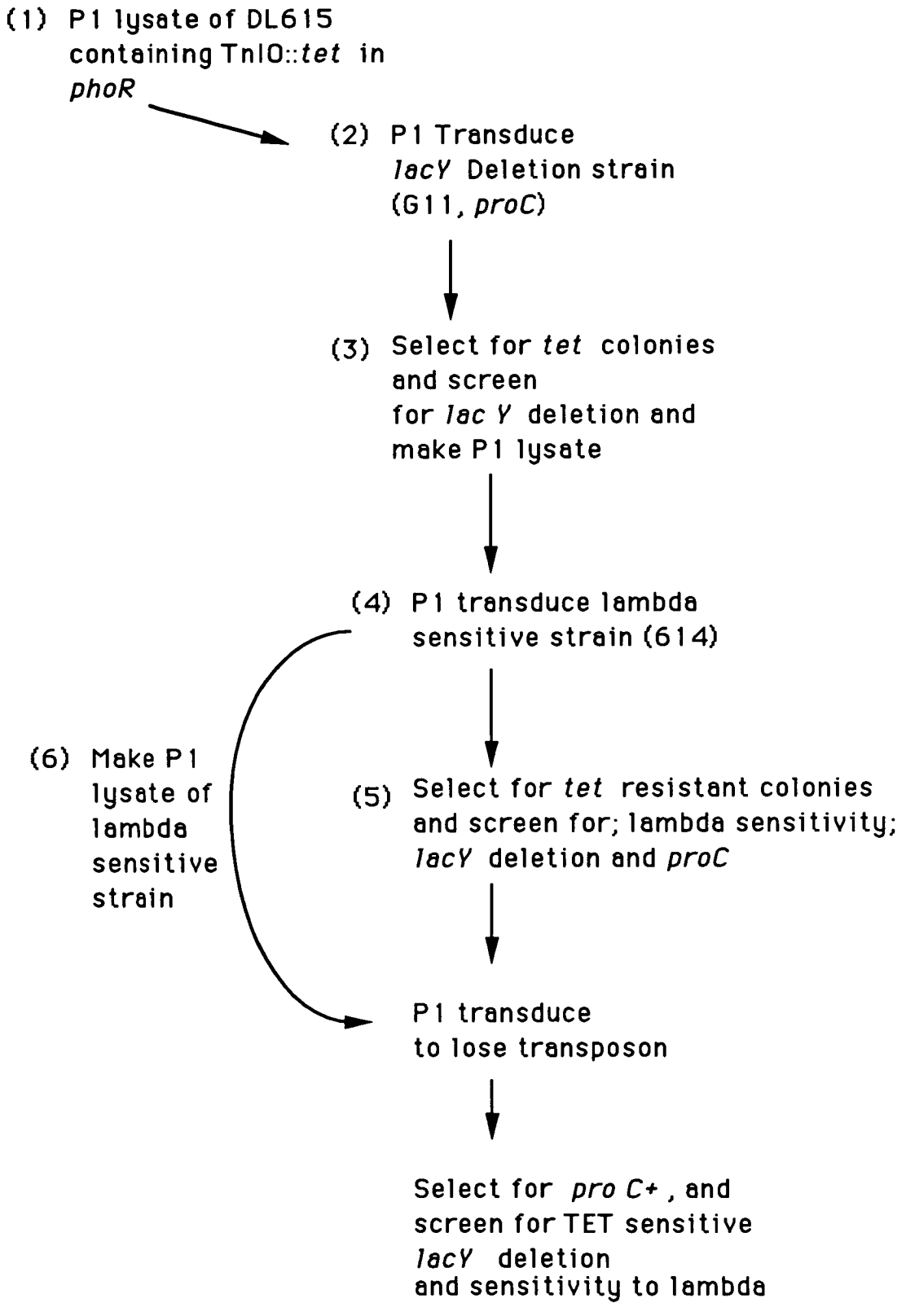
The transposon was removed by using the P1 lysate of NM614 to replace the *proC*$^-$ *tet*^{r} region with wild type DNA (Step (6) Figure 28). The transductants were selected as *proC*$^+$ colonies and subsequently screened for *tet*^{s} and for the presence of *lac*Δ *Y*. Of the *proC*$^+$ colonies isolated, 19% were *tet*^{s} and retained the *lac*Δ *Y* mutation.

Lysogens of the λ sensitive *lac*Δ *Y* strain, 614ΔY, were made with phage 1 and phage 2 as described in the methods Section 3.17.

Figure 28: Construction A λ Sensitive *lac*Δ *Y* Strain

An outline of the strategy employed for the transduction of the *lac*Δ *Y* from the λ resistant strain, G11, into a λ sensitive background, NM614.

Figure 28 :: Strategy For Transduction of *lac Y* Deletion to lambda Sensitive background



6.6 Preparation of lysogens in Strain 614ΔY with phage 1 and 2

The phage lysates of the constructs described in the previous section were used to prepare lysogens of the *lacY* deletion strain, 614ΔY, using the procedure described in the methods Section 3.17. Two cells only controls were carried out, plating out with and without the lytic phages (λNM63 and λNM220). The colonies produced were tested for lysogeny as described in the methods Section 3.17. The strain background was tested and also the *trp* phenotype by plating on minimal plates with/without supplements.

Initially *trp*$^-$ lysogenic colonies were screened for but none was found among the lysogens of both phages. This was thought to be due to the possible expression of the complete *trpB* gene in the lysogen from a phage promoter (Figure 29), and therefore only curing the cells of the prophage would result in the Trp$^-$ phenotype.

6.7 Curing of Strain 614ΔY Prophage

Bacteria were cured of prophage as described in the methods Section 3.18, re-purified and tested for the correct background and also for the Trp phenotype.

Of the cured colonies screened 6% were Trp$^-$ (Phage 1) and 10% (Phage 2). It appears that the phage with the greater homology (approximately 3kb on one side) generated more integrants. However it is not clear whether this is significant or not since although the extra homology may increase the fre-

quency of integration it may also increase the frequency of correct excision and consequently the *lacY* would be excised. Analysis of the purified lysogens and their ability to generate integrants would have yielded a more accurate estimate of the effect of increasing the homologous flanking regions. Having isolated the lysogen, produced by a recombination event in one of the flanking regions, the frequency of Trp$^-$ derivatives would then depend on the probability of the second recombination event occurring in the same or second flanking region. It is the ratio of the sizes of the two flanking sequences, therefore, that is important in determining the frequency with which a particluar lysogen generates integrants.

Several Trp$^-$ colonies were isolated and purified.

6.8 Analysis of Trp$^-$ Colonies Generated from Phage 1 and 2 Lysogens

The results of analysis of phage 1 and phage 2 Trp$^-$ derivatives are discussed below. The basal strain, 614*lac*Δ*Y*, and the parental lysogens were used as controls.

6.8.1 Functional Analysis of the Trp$^-$ Transductants

The *trp*$^-$ cured cells were plated on both TONPG/IAA and LAC/IAA plates to test for functional permease. The concentration of IAA and tryptophan were varied over a range of values independently to select for optimum expression

of the permease. The results are reported and discussed in the next Chapter 7.

6.8.2 Restriction Analysis of the Trp$^-$ Transductants

Genomic DNA was prepared from the Phage 1 lysogens and cured Trp+/- derivatives and restriction digests of the DNA were analysed by southern blotting as described in the methods Section 3.6.5. The 2.1kb EcoRI *lacY* fragment and fragments isolated from the *E.coli trp* region were used as probes, prepared as described in methods Section 3.7.2. The restriction maps of the *E.coli trp* region, lysogens and cured strains are diagrammed in Figure 29.

HindIII restriction digests of constructed strain 614(5)*trp*$^-$ (Figure 30), were probed with the 2.1kb EcoRI fragment containing *lacY* and show a band of approximately 6kb in both the lysogen track and the *trp*$^-$ track which is not present in the *lac*Δ *Y trp*$^+$ control track. The same bands are also present when the HindIII digests are probed with a *trpAB* HindIII fragment. This confirms the expected pattern of fragments (Figure 29 (a,i))

HindIII restriction digests of constructed strain 614(14)*trp*$^-$ (Figure 31) probed with the 2.1kb EcoRI fragment containing *lacY* show a band of approximately 6kb in both the lysogen track and the *trp*$^-$ track which is not present in the *lac*Δ *Y trp*$^+$ track. The same bands are also present when the HindIII digests are probed with a *trpAB* HindIII fragment. A smaller frag-

ment, seen in the trp^+ track corresponding to the wild type *trp* region, is also present in these tracks. This confirms the expected pattern of fragments (Figure 29 (c,i) or (c,ii)).

The HindIII digests of strain 614(14)trp^- were also probed with a *trpC* HindIII fragment as a control since the HindIII fragment containing the *trpC* gene (3kb) should not change for each track since the *trpC* region is unaffected by the integration event. A band at 3kb was present in each track as shown in Figure31(b).

EcoRI digests of the genomic DNA of strain 614(5)trp^- were probed with the 2.1kb *lacY* EcoRI fragment. The southern analysis of the EcoRI digests of the genomic DNA is shown in Figure 32. In both the lysogen and trp^- derivative a 2.1kb fragment was present. The integrated *lacY* fragment was absent in the $lac\Delta Ytrp^+$ genomic track, confirming the results expected from the restriction maps of the lysogens and cured cells (Figure 29 (a), (b) and (c)).

Figure 29: Restriction Maps Of Lambda Lysogens and Cured DNA

(a,i) A schematic representation of a Phage 1 integrant formed via a crossover in the *trpA* gene and a restriction map of the Phage 1 integrant. The expected restriction pattern for the HindIII/SalI subclone is highlighted following HindIII or EcoRI digestion of genomic DNA. Alterations in the restriction pattern of the *trp* genes has also been shown.

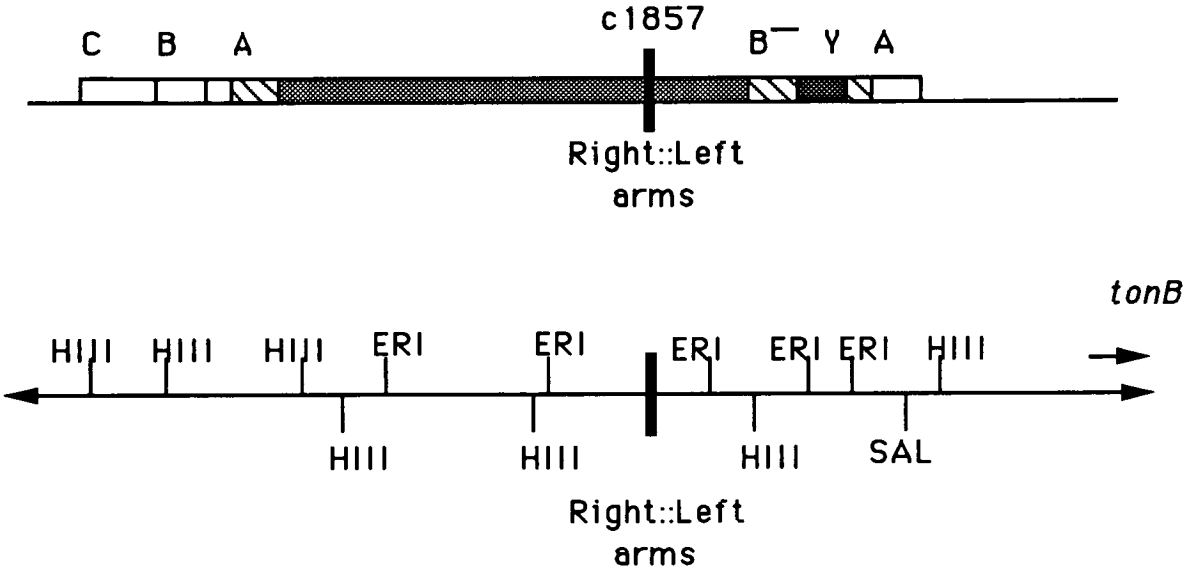
(a,ii) A schematic representation of a Phage 1 integrant formed via a crossover in the *trpB* gene and a restriction map of the Phage 1 integrant. The expected restriction pattern for the HindIII/SalI subclone is highlighted following HindIII or EcoRI digestion of genomic DNA. Alterations in the restriction pattern of the *trp* genes has also been shown.

(b) A schematic representation of the *E.coli trp* region of a cured lysogen generated from a second crossover event, for either Phage 1 or 2, and resulting in an integrated copy of *lacY* construct. The expected restriction pattern for the HindIII/SalI subclone is highlighted following HindIII or EcoRI digestion of the genomic DNA.

(c,i) A schematic representation of a Phage 2 integrant formed via a crossover in the *trpA* gene and a restriction map of the Phage 2 integrant. The expected restriction pattern for the HindIII/SalI subclone is highlighted following HindIII or EcoRI digestion of genomic DNA. Alterations in the restriction pattern of the *trp* genes has also been shown.

(c,ii) A schematic representation of a Phage 2 integrant formed via a crossover in the *trpB* gene and a restriction map of the Phage 2 integrant. The expected restriction pattern for the HindIII/SalI subclone is highlighted following HindIII or EcoRI digestion of genomic DNA. Alterations in the restriction pattern of the *trp* genes has also been shown.

Figure 29(a,i): : *Phage1* integrant formed with crossover in *trp A* gene



Hind III/Sall Fragment containing *lacY* construct

(4,606)

trpC *trpBA*

(2,777)(5,895)

HindIII Digest

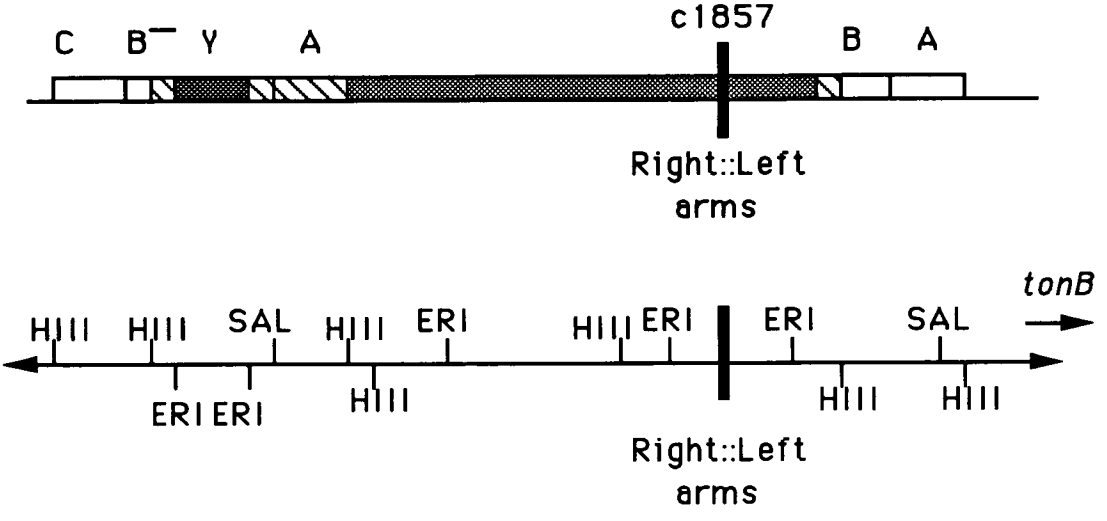
LacY insert

(5,352)

EcoRI Digest

(2,101)

Figure 29(a,ii):: Phage1 integrant formed with crossover in *trp B* gene



(4,606) Hind III/Sall Fragment containing *lacY* construct

trpC *LacY* insert

(2,777) (8,256)

HindIII Digest

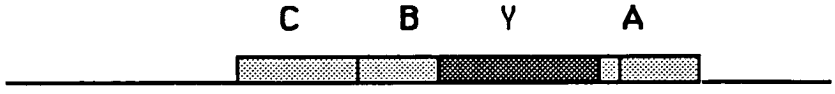
trpBA

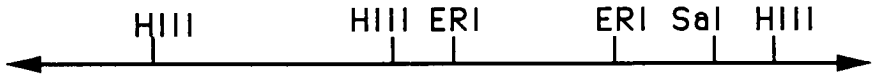
(2,963)

(2,101)

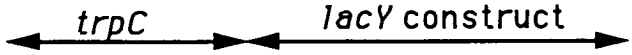
EcoRI Digest

Figure 29(b) :: Cured colonies



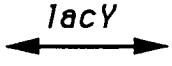


HinDIII Digestion



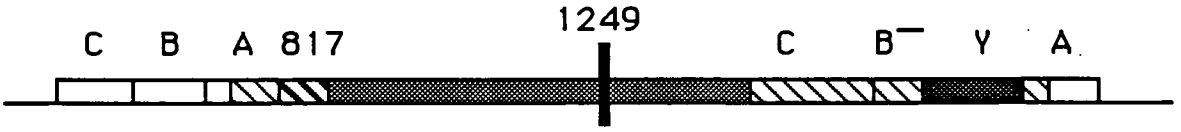
(2,777bp) (5,325bp)

EcoRI Digestion

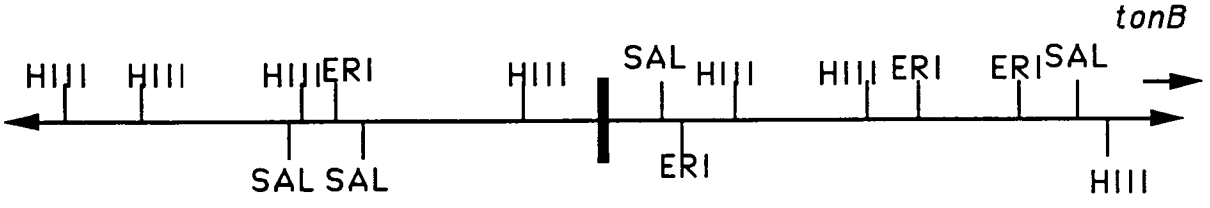


(2,101bp)

Figure 29(c,i):: Phage 2 integrant formed with crossover in *trp A* gene



Right::Left arms



Right::Left arms

Hind III/Sall Fragment containing *lacY* construct

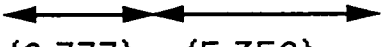
(4,606)

trpC *trpBA*

(2,777)(2,963)

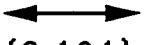
HindIII Digest

trpC *LacY* insert



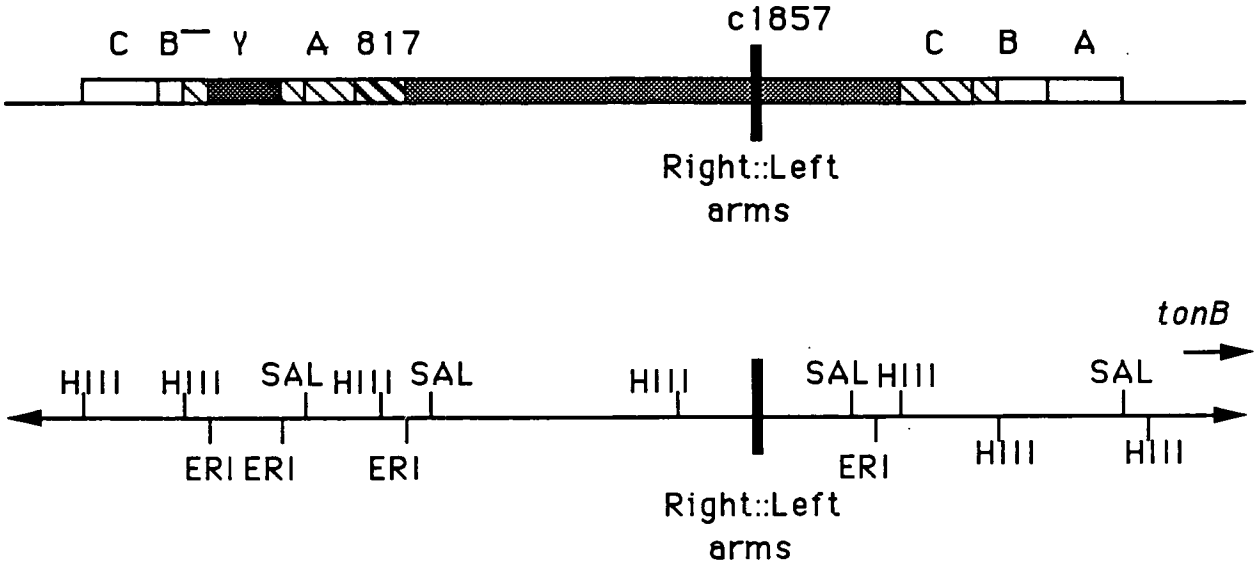
(2,777) (5,352)

EcoRI Digest



(2,101)

Figure 29(c,ii): : Phage2 integrant formed with crossover in *trp B* gene



Hind III/Sall Fragment containing *lacY* construct

(4,606)

trpC *LacY* insert

(2,777)(5,322)

HindIII Digest

trpC *trpBA*

(2,777) (2,963)

(2,101)

EcoRI Digest

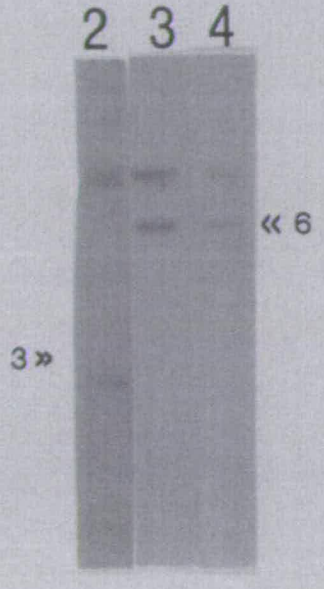
Figure 30: Southern Analysis of Phage 1 Trp^- Derivatives: HindIII Digests

(a)HindIII	Lane 1	λ HindIII Markers
	Lane 2	614*lac*Δ *Y*
	Lane 3	614(5)
	Lane 4	614(5)*trp*$^-$
(b)*trpAB* probe	Lane 2	614*lac*Δ *Y*
	Lane 3	614(5)
	Lane 4	614(5)*trp*$^-$
(c)*lacY* probe	Lane 2	614*lac*Δ *Y*
	Lane 3	614(5)
	Lane 4	614(5)*trp*$^-$

Figure 30

(a)

(b)



(c)

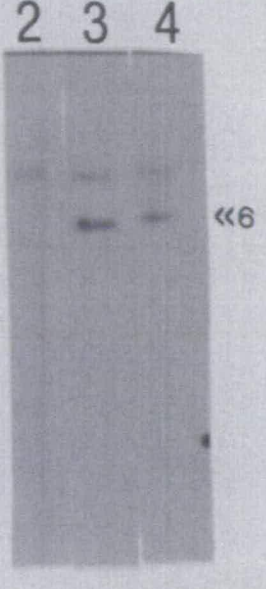
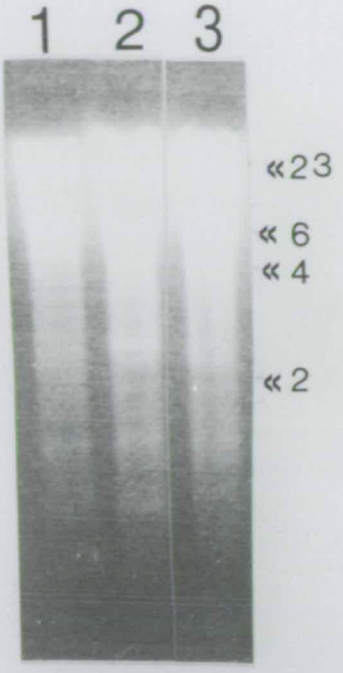


Figure 31: Southern Analysis of Phage 2 Trp$^-$ Derivatives: HindIII Digests

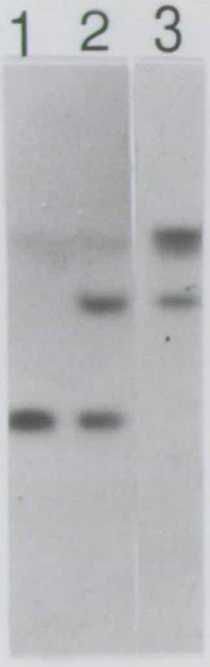
(a)HindIII	Lane 1	614$lac\Delta Y$
	Lane 2	614(14)
	Lane 3	614(14)trp^-
(b)$trpAB$ probe	Lane 1	614$lac\Delta Y$
	Lane 2	614(14)
	Lane 3	614(14)trp^-
(c)$lacY$ probe	Lane 1	614$lac\Delta Y$
	Lane 2	614(14)
	Lane 3	614(14)trp^-
(d)$trpC$ probe	Lane 1	614$lac\Delta Y$
	Lane 2	614(14)
	Lane 3	614(14)trp^-

Figure 31

(a)



(b)



(c)

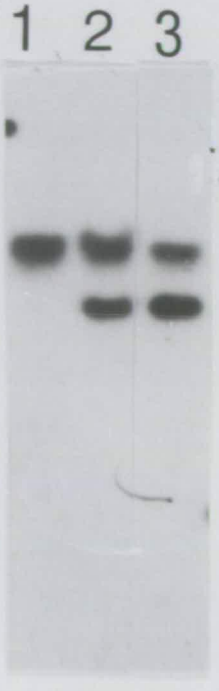


Figure 31
(d)

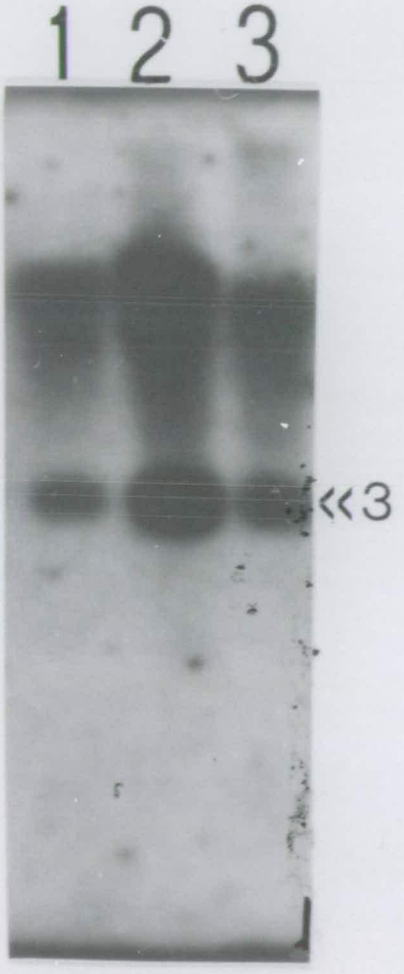
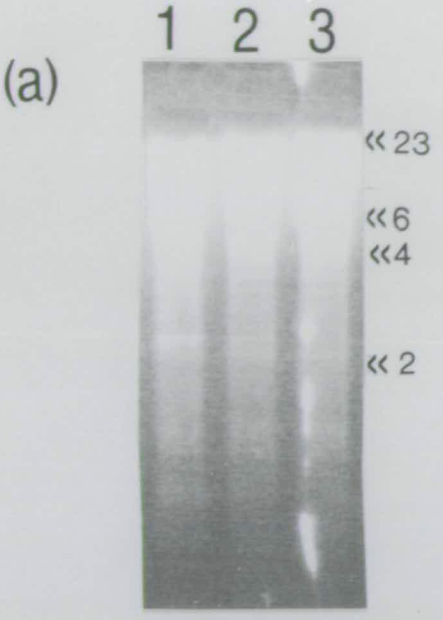
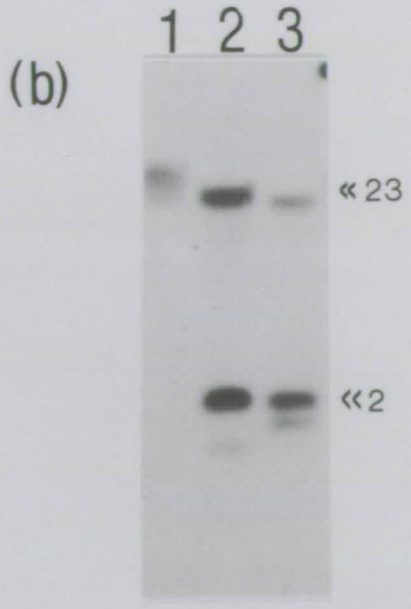


Figure 32: Southern Analysis of Phage 1 Trp$^-$ Derivative: EcoRI Digests

(a)EcoRI	Lane 1	614*lac*Δ *Y*
	Lane 2	614(5)
	Lane 3	614(5)*trp*$^-$
(a)*lac Y* probe	Lane 1	614*lac*Δ *Y*
	Lane 2	614(5)
	Lane 3	614(5)*trp*$^-$

Figure 32





6.9 Discussion

The genomic restriction digests of the various cell types confirmed that the desired strain had been constructed with the 2.1kb EcoRI *lacY* fragment under the control of the *S.marcescens trp* promoter integrated into the *trpB* gene in the *E.coli* chromosome.

This method of integration of a specific gene copy appears to be extremely efficient, particularly when the region of homology with the *E.coli* chromosome is large. Using phage λ allowed the selection of integration events, by selection of lysogens, and the isolation of cured bacteria, since these are viable at 42°C. These selection regimes were independent of the Trp phenotype of the colonies. Efficiency of the integration screening was greatly improved, thus allowing only cells which had undergone an integration event to be screened for their *trp* phenotype. Selection regimes, such as penicillin enrichment, could be avoided and the selection of spontaneous mutations would be minimal.

One spontaneous trp^- mutation was isolated during this procedure. This colony had also undergone an integration event but not at the *trp* locus, as demonstrated by hybrisation of genomic digests with the *trpAB* and *lacY* probes. This showed that although the 2.1kb fragment was present, the *trp* region did not vary in size as would be expected if the *lacY* construct had integrated there. This was confirmed on analysis of a sister trp^+ as this was also found to contain the *lacY* fragment.

The constructed strain was then used in expression studies to test the promoter and permease gene were functioning as required for the chemostat studies.

7 Studies of Expression of the Integrated *lacY* Construct

7.1 Introduction

The ideal system to apply MCA to the *lac* operon was one in which the genes of the operon were separated and controlled independently of one another. This was achieved by subcloning the *lacY* gene downstream of a suitable promoter i.e. a promoter capable of being induced over an equivalent range of expression to that of the *lac* promoter. The promoter chosen was that of the *trp* operon and the widest range of expression reported was that from the *trp* promoter of *S.marcescens*. The choice of the *trp* promoter also dictated that the basal strain have a trp^- mutation, in order to allow the full range of expression from the promoter.

It was also necessary to have a stable copy number of genes, best achieved by integration of a single copy of the *lacY* construct into the chromosome.

The resulting strain was then to be used in experiments in a chemostat with lactose as the limiting nutrient and the effect on growth of varying the two enzyme activities was to be measured. A profile of the physiological consequences of operon induction could then be produced.

Initial experiments carried out in batch culture have demonstrated that the requirements of the system, set in this thesis (Section 1.7), have been fulfilled. Growth on lactose and inducible permease activity were observed. Unfortunately, time did not allow any chemostat experiments to be carried out.

7.2 The Experimental System

7.2.1 The Experimental Strain

As demonstrated in the previous Chapter, the final construction was an E.coli strain, 614(5)*trp*$^-$ or 614(14)*trp*$^-$, which contained at the *lac* locus; the complete *lac* promoter/operator; the *lacZ* gene and the *lacA* gene. The *lacY* gene having been almost entirely deleted. At the *trp* locus, a copy of the *lacY* gene with the *S.marcescens trp* promoter/operator and attenuator sequences upstream is integrated into the *trpB* gene, rendering the strain *trp*$^-$.

7.2.2 Experimental Requirements of the Strain

The system set up in this thesis was designed specifically to allow the independent induction of the two *lac* genes, *Z* and *Y*.

It was, therefore, necessary to show that the constructed strain could

(1)Grow on lactose, as the sole carbon source.

(2)Induce a range of permease expression by varying the IAA concentration.

(3)Independently induce β-galactosidase and permease expression.

A series of experiments were carried out to demonstrate strain 614(5)*trp*$^-$ fulfilled all the requirements inherent in the system designed in this thesis. The expression of the *lacY* gene was examined initially and further to this, the independence of induction of the two genes was examined.

7.3 Permease Expression

7.3.1 Factors Affecting Permease Expression in Strain 614(5) *trp*$^-$

As discussed in Section 4.3 many factors contribute to *E.coli trp* operon expression. In a *trp*$^-$ strain background, like that of 614(5)*trp*$^-$, these differ slightly. Feedback inhibition of enzyme activity is no longer a factor since the level of tryptophan will be controlled externally. Only those factors which directly affect transcription were still thought to be important i.e repression and attenuation. Repression and attenuation work together to produce a wide range of *trp* expression in response to variable environmental conditions.

By maintaining the external concentration of tryptophan at as low a level as possible without affecting the growth rate, the levels of repression and attenuation could be set at constant values. Permease expression would then be induced using an antirepressor.

Only one of the constructed strains was used for experiments in expression, namely 614(5)*trp*$^-$.

7.3.2 Induction of Permease Expression in Strain 614(5)*trp*$^-$

Induction of the permease, under *trp* promoter control, was to be achieved by adding the tryptophan analogue, IAA, under mild tryptophan starvation conditions. IAA is a competitive antirepressor which binds to the same site on the aporepressor as tryptophan but does not induce the conformational

change to functional repressor, see Section 4.3.1. Using a *trp*$^-$ strain background a constant low level of intracellular tryptophan could be achieved, by maintaining the external tryptophan concentration at a constant level, and permease expression induced with differing concentrations of IAA. The low level of tryptophan is required to overcome the attenuation barrier as well as, in a *trp*$^-$ strain background, provide the tryptophan required for growth. Suitable or optimum concentrations of tryptophan and IAA have been found in previous expression studies, which cause a mild tryptophan deficiency, fully derepressing transcription but only inhibiting growth slightly.

The need for a low level of tryptophan has to be taken into consideration when designing experiments for the chemostat, since there can be only one limiting nutrient. If the level of tryptophan is such that it would limit the growth of the bacterial population then the concentration of lactose in the medium must be adjusted to ensure that it is limiting the rate of growth.

7.4 Assays for Functional *lac* Permease

Initial experiments were carried out on solid media and these were followed with growth experiments in batch culture and enzyme assays.

7.4.1 Solid Media Experiments

The constructed strain was tested for functional *lac* permease using the TONPG/IAA method. Initially, however, the effect of varying the concentration of

tryptophan only was examined using both minimal Succinate (S) and Lactose (L) plates. Subsequently, minimal lactose plates supplemented with inducer (LAC/IAA) were used in an attempt to find an optimum of expression before the TONPG test was applied.

The initial results obtained are shown in Tables 10, 11 and 12 and the TONPG/IAA results are shown in Table 13.

Several control strains were used to assess the different *lac* phenotypes; a wild type *lac* basal strain (614); a *lac*Δ *Y* strain derived from 614 (614*lac*Δ *Y*); the lysogenic parental strain (614(5)λ) and the two types of daughter strains i.e. $Trp^{+/-}$ cured cells. The Trp^{+} cured cells were Lac^{-} (*lac*Δ *Y*), having lost the integrated *lacY* construct and carrying the original *lac*Δ *Y*.

Table 10 shows that on succinate plates "starvation" conditions for tryptophan occur between 3 and 6μg/ml and growth did not improve until the tryptophan concentration was greater than 18μg/ml.

On lactose plates when the concentration of tryptophan was varied, as shown in Table 12, some growth of the 614(5)*trp*$^{-}$ was observed at the lower range of tryptophan concentrations i.e. 2-6μg/ml, which corresponds to the "starvation" conditions found in the Succinate growth experiments. Growth on lactose at these concentrations suggests that *trp* promoter expression was derepressed, and consequently some functioning permease was being expressed. The lysogenic strain did not show any growth under the same conditions,

perhaps due to interference of stronger phage λ promoters.

The tryptophan concentration chosen for the functional assay was 6μg/ml which was estimated to be sufficient to sustain reasonable growth but low enough to allow induction to occur.

With the addition of inducer, IAA, shown in Table 12, growth of the 614(5)*trp*$^-$ strain was observed over a wide range of IAA concentrations.

It was not possible to increase the concentration of inducer indefinitely, since concentrations greater than 400μg/ml had a deleterious effect on growth of the wild type control strain as well as 614(5)*trp*$^-$. IAA is known to compete with tryptophan in tRNA binding [11, 4, 69] and therefore would affect growth negatively, opposing the inductive effect on lactose entry. At high concentrations it can be lethal. The constructed strain appeared to be more sensitive to high IAA concentrations than the wild type (614). While the wild type has its high endogenous *trp* synthesis, as well as the added supplement, the dependence of the constructed strain depended only on the low levels of tryptophan set in the medium.

Having observed some functional permease expression on lactose minimal plates the optimised conditions were then applied to the TONPG assay as a further test: a *lac* constitutive strain, 6947, and a *lacY*$^-$ strain, NE4, together with the control strains mentioned in the previous experiments.

The concentrations of IAA and tryptophan used were 1.6μg/ml and 6μg/ml

respectively. The highest concentration of IAA tested which did not affect growth was used to allow the "maximum" permease expression. The results in Table 13 show a reduction in the growth of 614(5)*trp*$^-$ in the presence of TONPG/IAA/TRP which was not demonstrated in the presence of TONPG/IPTG/TRP, suggesting that there was an effect on the growth.

Again there was no positive control available for this test and it was difficult therefore to have absolute confidence in these results, particularly since the assessment of growth was subjective. However the ability of the control strains to grow in the presence of IAA suggests that it is not affecting cell growth and therefore the reduction in growth observed with TONPG/IAA/TRP is due to the effect of the TONPG and the expression of permease. The observation of only a reduction in growth, and not lethality, was believed to be due to the level of permease expression being lower than that of a wild type *lac* strain. The TONPG was not able to reach sufficiently high levels intracellularly to kill the cells.

It was clear from the solid media results that functional permease was being expressed in the constructed strain, 614(5)*trp*$^-$. Growth was observed on minimal lactose plates supplemented with tryptophan only, and with tryptophan and antirepressor, IAA. An optimised ratio of tryptophan to IAA (6μg/ml to 1.6μg/ml respectively) was obtained which gave growth on lactose comparable to wild type. Complete lethality was not observed with the constructed strain

in the TONPG assay but a small reduction in growth was observed.

Table 10: Effect of Variation of Tryptophan Concentration on Growth on Minimal Succinate (S) Plates

TRP (μg/ml)	Strain 614	614*lac*ΔY	614(5)λ	614(5)*trp*$^+$	614(5)*trp*$^-$
0	+++	+++	-	+++	-
3	+++	+++	-	+++	-
6	+++	+++	+	+++	+
18	+++	+++	++	+++	++
21	+++	+++	+++	+++	+++
50	+++	+++	+++	+++	+++

Table 11: Effect of Variation of Tryptophan Concentration on Growth on Minimal Lactose (L) plates

TRP (μg/ml)	Strain 614	614*lac*ΔY	614(5)λ	614(5)*trp*$^+$	614(5)*trp*$^-$
0	+++	-	-	-	-
2	+++	-	-	-	+
4	+++	-	-	-	++
6	+++	-	-	-	++
10	+++	-	-	-	+
50	+++	-	-	-	+

Table 12: Effect of Variation of IAA Concentration on Growth on Minimal Lactose(L) Plates Supplemented with Tryptophan (6μg/ml)

IAA	Strain				
μg/ml	614	614*lac*ΔY	614(5)λ	614(5)*trp*$^+$	614(5)*trp*$^-$
0	+++	-	-	-	+
0.04	+++	-	-	-	++
0.4	+++	-	-	-	++
1.6	+++	-	-	-	+++
8	+++	-	-	-	++
16	+++	-	-	-	++
40	+++	-	-	-	+
400	+	-	-	-	-

Table 13: Selection Against *lacY*$^+$ Colonies Using TONPG and IAA

	Strain				
Min (S)	614	614*lac*ΔY	6947	NE4	614(5)*trp*$^-$
TRP (6μg/ml)	+++	+++	+++	+++	+++
TONPG/IPTG	-	+++	-	+++	-
TONPG/IPTG/TRP	-	+++	-	+++	+++
IAA (1.6μg/ml)	+++	+++	+++	+++	-
TONPG/IAA	+++	+++	-	+++	-
TONPG/IAA/TRP	+++	++++	-	++++	++

7.5 Batch Culture Experiments

The optimised conditions obtained from the solid media experiments, described in the previous section i.e. 1.6μg/ml IAA and 6μg/ml tryptophan, were then translated to batch culture experiments in order to more accurately measure the growth of the strains and assay for permease activity.

7.5.1 Growth of 614(5)*trp*$^-$ On Lactose as the Sole Carbon Source

Glucose, succinate and lactose were used as carbon sources and batch culture experiments were carried out as described in the methods Section 3.22. The initial inoculum was approximately 10^7 cells/ml and OD_{600} of the culture was monitored as a measure of growth. As with the solid media several control strains were used, a wild type strain (614), a predecessor strain carrying the *lac*ΔY and a sister strain also cured for phage λ but with no subsequent integration (*trp*$^+$). All of the strains grew normally on glucose, the wild type strain growing slightly better than the recombinant strain which was thought to be due to the tryptophan requirement. Growth on succinate was observed and was considerably slower than that on glucose. Succinate being a poorer carbon source than glucose, this was expected.

The growth curves are shown in Figure 33. Growth on lactose was observed with strain 614(5)*trp*$^-$ and was slower than that observed with the wild type control. Batch culture experiments, with glucose as the sole carbon source,

with varying levels of tryptophan had previously demonstrated that 6μg/ml tryptophan did not affect growth rate over the time scale used in these experiments.

The reduction in growth of 614(5)*trp*$^-$ may have been due to the level of tryptophan being lower than was required when in competition with IAA.

Increasing the tryptophan concentration from 6 to 30μg/ml while maintaining the ratio of tryptophan to IAA did allow the accumulation of more biomass in the cultures (Figure 33 (b)).

7.5.2 Variation in Levels of Induction of Permease with IAA in 614*trp*$^-$

Growth experiments on lactose, in which the IAA/TRP ratio was altered, were then carried out. The variation was limited to the range of IAA and tryptophan used in the solid media experiments. The tryptophan concentration used was 3.2μg/ml. This was less than used previously since the time period for the batch culture experiments was less than that for the solid media experiments. Using the lower concentration of tryptophan was hoped to generate greater permease expression.

Initially, therefore, the tryptophan concentration was held constant at 3.2μg/ml and the concentration of IAA varied between 1-48μg/ml. The growth curves are shown in Figure 34. As the level of IAA increased some detrimen-

tal effect on the growth was observed; above 16μg/ml the growth is reduced. Reducing the level of IAA below 8μg/ml produced the highest level of growth. Growth began to decline below 4μg/ml.

The ratio of IAA to tryptophan which gave the best results i.e. allowed growth of 614(5)*trp*$^-$ approximating that of the wild type strain on lactose, was found to be 8μg/ml IAA to 3.2μg/ml tryptophan.

Growth of the constructed strain 614(5)*trp*$^-$ was observed in minimal lactose medium in batch culture experiments. The ratio of tryptophan to IAA obtained from the solid media experiments did not generate the best growth. Maintaining the ratio but increasing the initial concentration did give growth comparable to that of the wild type strain. Some variation in growth was observed with respect to variation in IAA but the differences observed in the growth rate, with respect to changes in IAA concentration, were relatively small. At this stage it was thought that the induced changes in permease expression would be more accurately measured using a permease assay rather than the ability to grow on lactose in batch culture.

Figure 33: Growth of Strain 614(5)trp^- on Minimal Lactose supplemented with IAA and Tryptophan

(a) Growth of strain 614(5)trp^- relative to wild type, NM614 and a $lac\Delta Y$ strain, 614$lac\Delta Y$ in lactose minimal medium supplemented with 1.6μg/ml IAA and 6μg/ml tryptophan. The concentrations of IAA and tryptophan used were adapted from the solid media growth experiments.

(b)Growth of strain 614(5)trp^- relative to wild type, NM614 and a $lac\Delta Y$ strain, 614$lac\Delta Y$ in lactose minimal medium supplemented with 8μg/ml IAA and 30μg/ml tryptophan. The concentrations of IAA and tryptophan used were optimised to increase the biomass of the culture and to obtain a growth curve similar to that of the wild type.

Figure33(a)::Growth of Strain 614(5)trp- on Minimal Lactose supplemented with IAA and tryptophan

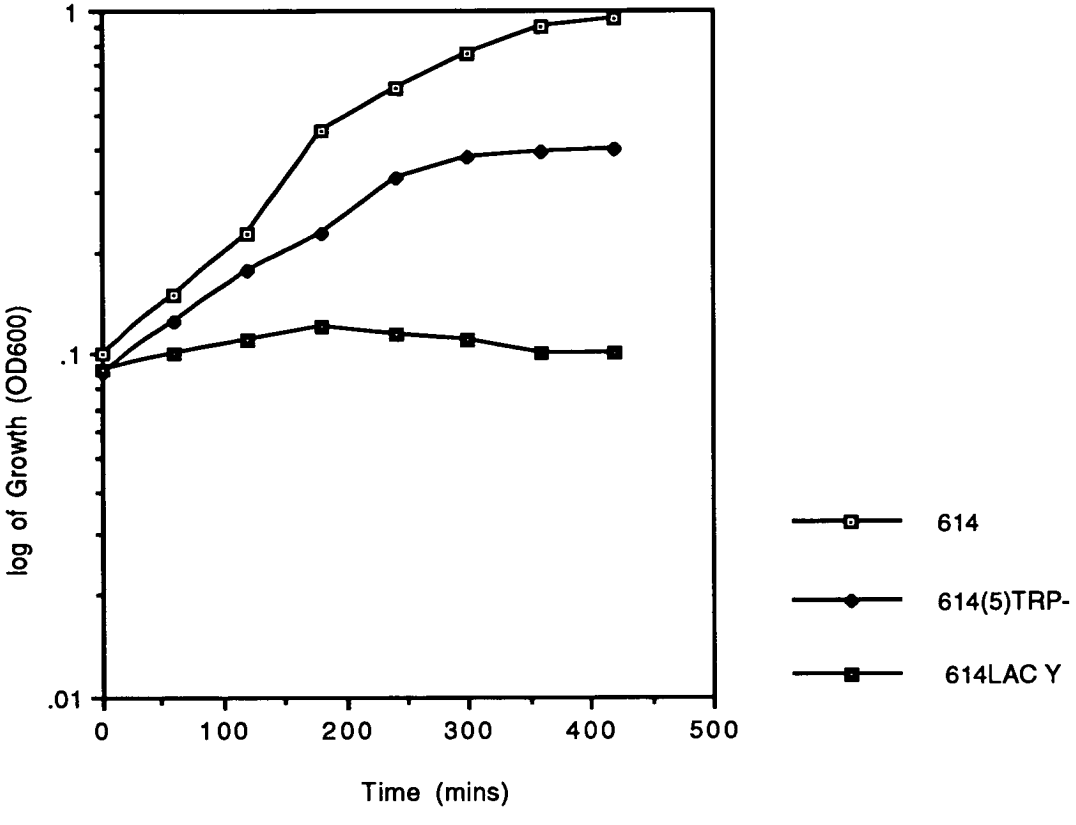


Figure 33(b):: Growth of Strain 614(5)trp- on Minimal lactose supplemented with IAA and tryptophan

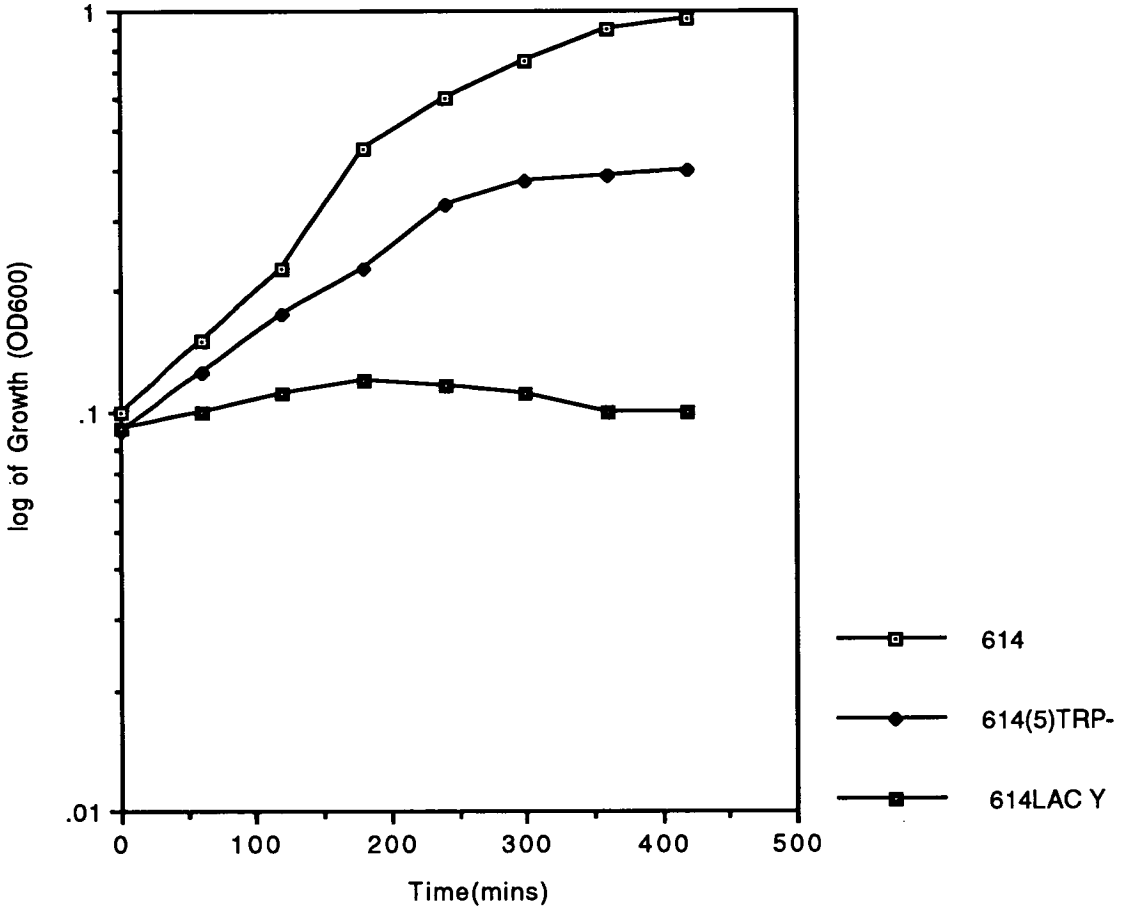
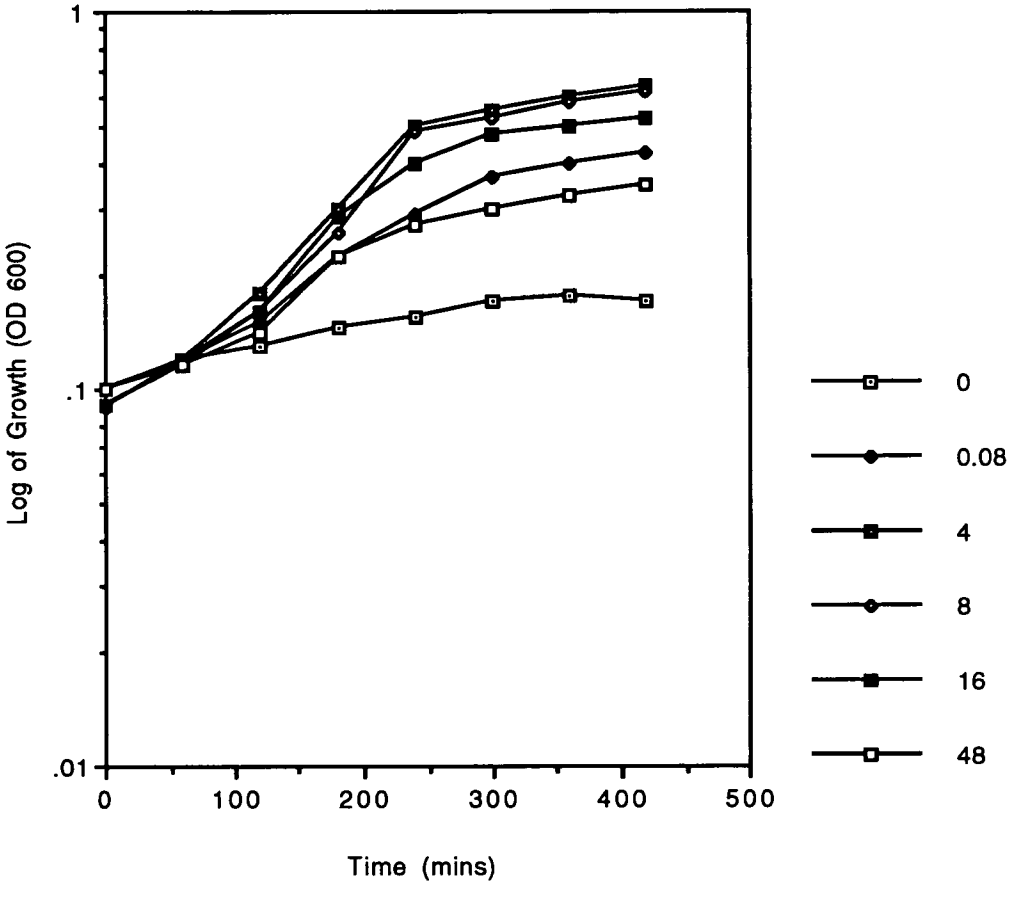


Figure 34: Growth of Strain 614(5)trp^- on Minimal Lactose supplemented with varying concentrations of IAA

Growth of strain 614(5)trp^- relative to wild type, NM614 and a $lac\Delta Y$ strain, 614$lac\Delta Y$ in lactose minimal medium supplemented with 3.2μg/ml tryptophan. The concentration of IAA was varied between 1-48μg/ml.

Figure34:: Growth on Lactose with varying IAA concentrations



7.5.3 Permease Enzyme Assay

In order to demonstrate that the growth observed by the constructed strain, on lactose, was due to induction of permease, and not due to diffusion of lactose into the cell, permease activity was assayed. A simple assay was used, which monitored the rate of uptake of ONPG and its subsequent conversion to ONP in an intact culture of cells [65, 25] and is described in the methods Section 3.24.2. The cells were grown in minimal succinate medium and β-galactosidase was fully induced using IPTG (10^{-3}M). The level of permease was set using differing levels of IAA. The rate of conversion to ONP was assumed to be proportional to rate of uptake of the galactopyranoside.

The strains were grown as described in methods Section 3.22 and the enzyme activity was calculated in units per min per bacterium as described in the methods Section 3.24.2. A wild type *lac* strain, 614, and a *lac*ΔY strain, 614*lac*ΔY, were used as controls.

Table 14: Effect On Permease Expression Of Varying Tryptophan Concentration

(x 10^{-3})

TRP (μg/ml)	Enzyme Activities (u/min/bacterium) β-galactosidase	Permease
0	2753.2	14.1
0.5	ND	25.6
1.0	ND	22.1
2.5	ND	16.4
12.5	ND	19.6
50	3256.2	3.4

Table 15: Permease Activities of Strain 614(5)*trp*$^-$ with differing concentrations of IAA and Low Tryptophan (3.2μg/ml)

(x 10^{-3})

IAA (μg/ml)	Enzyme Activity (u/min/bacterium) 614	614*lac*ΔY	614(5)*trp*$^-$
0	517.4	-	10.4
0.4	ND	-	25.0
0.8	ND	-	103
8	ND	-	220
16	ND	-	83.5
32	535.7	-	60.4
48	750.0	-	47.8

Table 16: Permease Activities of Strain 614(5)trp^- with differing concentrations of IAA and Excess Tryptophan (30μg/ml)

$\times 10^{-3}$

IAA (μg/ml)	Enzyme Activity (u/min/bacterium)		
	614	614$lac\Delta Y$	614(5)trp^-
0	153.5	-	3.4
0.4	ND	-	3.6
0.8	ND	-	23.7
8	ND	-	47.6
16	ND	-	54.3
32	ND	-	18.18
48	128.9	-	ND

7.5.4 Variation in Tryptophan Concentration

An initial experiment was carried out in which only the tryptophan concentration was varied, to assess at which point the *trp* promoter was fully switched off. A basal level of permease expression was measured throughout, which was reduced with high concentrations of tryptophan to negligible level.

It is clear from the results (Table 14) that the level of tryptophan required to fully repress *trp* promoter expression is considerably higher than expected. The concentration of tryptophan required to achieve full repression would be such that the levels of IAA necessary to induce expression would need to be greatly increased. The basal level of expression was considered low enough not to interfere with the assay significantly.

7.5.5 Variation In IAA Concentration

The tryptophan concentrations used were 50μg/ml, to generate an excess and 3.2μg/ml for low concentrations. The concentrations of IAA used to induce permease for the enzyme assays were based on the growth experiments on lactose and varied from 0 to 48μg/ml.

The consistency of results was poor from experiment to experiment, the measured enzyme activities showing up to ten fold variation, although within experiments the results were consistent, the maximum deviation being +/- 13%. The induction profile always followed the same pattern but the level

of enzyme activity varied considerably. This was possibly due to variation in the concentration of tryptophan remaining in the culture after washing and variation in inoculum size.

Tables 15 and 16 show the activities measured in cultures of 614(5)*trp*$^-$ under varying IAA concentrations from one experiment. In Table 15, with low levels of tryptophan, the range of permease activity seen is between 220 and 10.4 units. The activity over this range was not linear with increasing amounts of IAA. A fall off in activity was observed above 8μg/ml IAA of inducer. A basal level of expression was observed in most experiments which was thought to be a result of the low tryptophan concentrations used in the assay. However in experiments where excess tryptophan was added to the culture, an example of which is shown in Table 16, a basal level of expression was still present although reduced in magnitude.

Inducible permease expression, of approximately 20 fold, was observed in the constructed strain. An important factor in obtaining variation of permease expression was found to be the concentration of tryptophan in the medium. As a consequence, in cultures with high concentrations of tryptophan added (50μg/ml), the level of permease expression achieved with the addition of IAA was much less than in cultures with low levels of tryptophan added.

7.6 Independent Induction of the *lac* Genes

The assay for permease activity relied on activity of β-galactosidase to convert ONPG to ONP inside the cell. The experiments described above were therefore carried out with a constant level of IPTG (10^{-3}M) in the medium to ensure a constant level of β-galactosidase. The activity of β-galactosidase was also assayed using ONPG as described in the methods Section 3.23 but the cells were lysed before carrying out the assay. Typically, in the assays for permease, the β-galactosidase activity was 3000units/min/bacteria. $\times 10^{-3}$ A series of experiments were carried out to demonstrate independent induction. The cultures were grown as described in the methods Section 3.22. The permease was set at a constant level using IAA and tryptophan concentrations of 8μg/ml and 3.2μg/ml respectively and β-galactosidase expression was varied using different concentrations of IPTG. The results are shown in Table 17.

β-galactosidase activity increased with IPTG concentration while permease activity remained constant. A drop in permease activity was observed when no IPTG was added. This was thought to be an artefact of the permease enzyme assay, the induced level of β-galactosidase was so low that it interfered with the permease assay.

Independent induction of the two *lac* genes was observed. Permease activity was held at a constant level while β-galactosidase activity was varied over a wide range of activities.

Table 17: Independent Induction of β-galactosidase and Permease in Strain 614(5)trp^-

$(\times 10^{-3})$

IPTG μg/ml	Enzyme Activities u/min/bacterium	
	β-galactosidase	Permease
0	3.2	9.42
1.25	8.9	ND
2.5	10.1	95.4
5	367	81.9
12.5	1039	96.6
50	2752	84.31
125	3667	ND
250	5074	85.7

7.7 Discussion

From the experiments described above it can be concluded that the experimental system outlined in the objectives of this thesis has been successfully set up.

The constructed strain was observed to grow on lactose solid and liquid media in the presence of IAA and low levels of tryptophan. Permease activity was also measured and variation in the actitvity was observed with different IAA concentrations. Finally, the induction of the permease activity was shown to be independent of the β-galactosidase activity and it was also demonstrated that induction of β-galactosidase was independent of permease activity.

Difficulties in handling the competition between tryptophan and IAA have not allowed the system to reach its full potential in the batch culture experiments carried out. It would have been preferable, if time had allowed, to use the chemostat for these experiments. The difficulty in controlling the ratio of IAA and tryptophan in batch experiments would have been much less of a problem in the chemostat. It would be possible in chemostat experiments to set the ratio at the start of the experiment and it would be held constant throughout. Unfortunately, in the batch experiments the tryptophan in the starting medium is depleted as the cells grow and therefore the ratio of IAA: tryptophan increases as the experiment continues. The time point, therefore, at which the culture is assayed for permease activity is critical i.e. such that

the ratio of IAA : tryptophan is constant at the point of assay.

Future work with this system would necessarily involve chemostat experiments. Having established optimum conditions for permease expression in the chemostat, it would be possible to study the physiological consequences of *lac* operon expression in much more detail, by obtaining a more complete set of data.

8 Summary

The immediate aims of the project were set out in the introduction, namely;

(1) to separate the *lacZ* and *lacY* genes of the of the lactose operon such that they could be controlled/ induced independently

(2) to maintain the new construct stably in the *E.coli* chromosome

The *lacY* gene was subcloned into plasmid PBN372 such that it was downstream of the *S.marcescens trp* promoter. The flanking *E.coli trp* genes were exploited, via homologous recombination to integrate the construct into the *E.coli* chromosome at the *trpB* locus.

Two approaches were employed to achieve integration;

(1) transformation with a suitable recipient *recD* strain

(2) transduction with phage λ

The first method was unsuccessful, only spontaneous trp^- mutations were isolated. The second method yielded several integrants, one of which was iused in subsequent growth experiments.

The constructed strain was, as a consequence of the integration event, rendered trp^-. This allowed the tryptophan concentration in the medium to be maintained at a constant level and the level of iduction to be set by the addition of differing amounts of the antirepressor, IAA. Growth of the constructed strain in minimal lactose media was observed which was comparable with wild

type *E.coli*. Permease activity of the constructed strain was seen to vary when assayed in the presence of varying amounts of IAA. The expression of permease was also demonstrated to be independent of β-galactosidase activity.

The constructed strain therefore met all the initial requirements of the experimental system set out in this thesis.

The difficulties encountered during the analysis due to the competition between antirepressor (IAA) and corepressor (tryptophan) would have been minimal had the analysis been carried out in chemostat experiments. The level of tryptophan would then have been constant throughout the experiment which is not thought not possible in batch culture, since the initial level of tryptophan is relatively high and is depleted as the experiment progresses.

A Appendix

.1 Growth Media

.1.1 Liquid Media

Luria Bertani LB (1l)

10g Bacto-tryptone

5g Bacto-yeast extract

10g NaCl

M9 Medium 10x (1l)

30g Na_2HPO_4

15g KH_2PO_4

2.5g NaCl

1g NH_4Cl

Adjusted to pH 7.4, autoclaved before adding the following, which have been sterilised separately.

2mls 1M $MgS0_4$

10mls 20% Glucose

0.1ml 1M $CaCl_2$

Hobson's 10x (1l)

105g K_2HPO_4

10g tri sodium citrate

45g KH_2PO_4

10g NH_4SO_4

The solution was autoclaved before adding the following, which had been sterilised separately.

	10mls 0.1M $MgSO_4$
	10mls 20% Sugar
A medium 5x (1l)	52.5g K_2HPO_4
	22.5g KH_2PO_4
	5.0g NH_4SO_4
	2.5g tri sodium citrate
SOB (1l)	20g Bacto-tryptone
	5g Bacto-yeast extract
	10mls 1M NaCl
	2.5mls 1M KCl
	10mls 1M $MgSO_4$
	10mls 1M $MgCl_2$

The solution was sterilised, by autoclaving, in the absence of the $MgSO_4$ and $MgCl_2$ which were added immediately prior to use.

SOC (1l) As SOB but containing 20mls 1M Glucose.

TSS(1l)	100g PEG 8000 (10% wt/vol)
	50mls DMSO (5% vol/vol)
	20-50mls 1M $MgCl_2$ (or 1M $MgSO_4$)
	↑ 900mls with LB medium

The pH was adjusted to 6.5 and made up to 1l with more LB medium.

.1.2 Solid Media

The LB and Minimal medium were made according to the above formulae. Prior to autoclaving agar/ agarose was added as follows;

Bacto-agar/Agarose	15g (for 1l of plates)
	7g (for 1l of Top-agar)
LM plates (1l)	10g Bacto-tryptone
	5g Bacto-yeast extact
	10mls 1M NaCl
	10mls 1M $MgSO_4$
	15g Bacto-agar

.1.3 Antibiotics

The concentrations of antibiotics used were given in Maniatis [76] and Miller [54].

.2 Buffers

Phosphate buffer 1M (ph 7.0)	61.0mls 1M Na_2HPO_4
	39.0mls 1M NaH_2PO_4
IPTG	25mg
	1ml DMF

IAA	5mg
	1ml DMF
TONPG	was added as solid where required.

.2.1 Electrophoresis Buffers

Tris Acetate 50x (1l)	242g Tris base
	57.1ml Glacial acetic acid
	100ml 0.5M EDTA (pH 8.0)
Tris Borate 10x (1l)	108g Tris base
	55g Boric acid
	20ml 0.5M EDTA (pH 8.0)
Loading Buffer	20% Ficoll
	0.2% Bromophenol Blue
Resolving Gel Buffer	22.7g Tris base (Sigma T1378)

Made up to 50mls with dH_2O, pH to 8.7 with HCl and made up to 100ml with dH_2O.

Stacking Gel Buffer	15.14g Tris base (Sigma T1378)

Made up to 50mls with dH_2O, pH to 6.75 with HCl and made up to 100mls with dH_2O.

Running Gel Buffer (2l)

6.05g Tris base (Sigma T1378)

2.00g SDS

29.10g Glycine

.3 DIG Buffers

Buffer 1 10x

1M Maleic Acid

1.5M NaCl

and the pH was adjusted to to 7.5 with NaOH.

10% Block (100mls)

10% Blocking Reagent

1 x Buffer 1

Washing Buffer

0.3% Tween-20

1 x Buffer 1

Buffer 2

1% Blocking Reagent

1 x Buffer 1

Buffer 3 (10x)

1.0M Tris.Cl

1.0M NaCl

0.5M $MgCl_2$

adjust the pH to 9.5 using NaOH.

.4 DNA preparation solutions

.4.1 Genomic DNA

TEL (500μl)	5mg lysozyme
	20μl 1M Tris (pH 8.0)
	20μl 0.5M EDTA (pH 8.1)
	460μl H_2O
SDS/RNAse solution (100 μl)	25μl 20% SDS
	1μl 10mg/ml RNAse
	74μl H_2O

.4.2 Plasmid DNA

Solution I	50mM Glucose
	25mM Tris.Cl(pH 8.0)
	10mM EDTA
Solution II	0.2N NaOH
	1% SDS
Solution III (100mls)	60.0mls 5M KAc
	11.5mls Glacial Acetic acid
	28.5mls H_2O

.4.3 Phage DNA

PEG Solution 20% PEG (6,000)

2.5M Nacl

.5 Other Solutions

30% Acrylamide (100ml) 29.2g Acrylamide

0.8g Bis-acrylamide

1M Dithiothreitol DTT 3.09g DTT

20mls 0.01M NaOAc (pH 5.2)

The solution was sterilised by filtration and dispensed into aliquots of 1ml and stored at -20°C.

SSC 20x (1l) 175.3g NaCl

88.2g Sodium citrate

The solution was made up to 800mls with dH_2O, the pH was adjusted to 7.0 with a few drops of 10N NaOH and the volume was then made up to 1l . The solution was sterilised by autoclaving.

SSPE 20x (1l) 174g NaCl

27.6g NaH_2PO_4

7.4g EDTA

The solution was made up to 800mls with dH_2O, the pH was adjusted to 7.4 with 10N NaOH and the volume was the made up to 1l. The solution was sterilised by autoclaving.

TE (pH 7.4)	10mM Tris.Cl(pH 7.4)
	1mM EDTA(pH 8.0)
TE (pH 7.6)	10mM Tris.Cl (pH 7.6)
	1mM EDTA (pH 8.0)
TE (pH 7.8)	10mM Tris.Cl (pH 7.8)
	1mM EDTA (pH 8.0)
Nick Translation Buffer (10x)	0.5M Tris.Cl(pH 7.2)
	0.1M $MgSO_4$
	1mM Dithiothreitol
	500μg/ml BSA (Pentax Fraction V)

Deoxynucleotide Triphosphates

The NTPs or dNTPs were dissolved in sterile distilled water directly in the shipping bottle at an expected concentration of 10mM and the pH was adjusted to pH 7.0 using a dilute solution (0.05M) of Tris base. The neutralised solution was then aliquoted and diluted to the appropriate concentrations. The exact

concentrations were calculated by reading the optical density at the wavelength given and using the values for the extinction coefficients in the table.

Nucleotide	**Wavelength**	**Extinction Coefficient** (1/M cm)
A	259	1.5×10^4
G	253	1.37×10^4
C	271	9.10×10^3
U	262	1.00×10^4
T	260	7.40×10^3

N.B. For a cell of 1cm Absorbance=ϵ/M , where ϵ is the extinction coefficient and M is molarity.

TFB

10mM K-MES (pH 6.2)[1]

100mM RbCl (or KCl)[2]

45mM $MnCl_2$

10mM $CaCl_2$

3mM Hexamine cobalt (III) chloride.

All the salts were added as solids and the solution was sterile filtered and stored at 4°C. This solution is stable for up to a year when stored at 4°C.

Nitrocefin (1ml)

5.0mg Nitrocefin

0.5ml DMSO

Immediately the compound has dissolved, 9.5mls of 0.1M phosphate buffer (pH 7.0) was added and shaken well. The solution can be stored in the dark at 4°C for up to 14 days.

Z Buffer (1l)

16.1g $Na_2HPO_4.7H_2O$

5.5g $NaH_2PO_4.H_2O$

0.75g KCl

0.246g $MgSO_4.7H_2O$

2.7ml β-mercaptoethanol

The solution was not autoclaved.

.6 DNA Molecular Weight Markers

The molecular weight markers were obtained from Boehringer Mannheim.

λ**HindIII**: 23.1kb;9.4kb; 6.6kb; 4.4kb; 2.3kb; 2.0kb; 0.56kb.

1kb Markers:12kb; 11kb; 10kb; 9kb; 8kb; 7kb; 6kb; 5kb; 4kb; 3kb; 2kb; 1.5kb; 1.0kb; 0.5kb; 0.4kb; 0.3kb; 0.2kb.

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